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# **Targeted Biomarker Quantitation by LC–MS**

*Edited by*

*Dr. Naidong Weng and Dr. Wenying Jian*

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## List of Contributors

***Bo An***

Department of Pharmaceutical Sciences  
 School of Pharmacy and Pharmaceutical Sciences  
 Buffalo, NY  
 USA and  
 New York State Center of Excellence in Bioinformatics  
 and Life Sciences  
 Buffalo, NY  
 USA

***Ray Bakhtiar***

Teva Branded Pharmaceutical Products R&D, Inc.  
 West Chester, PA  
 USA

***Michael G. Bartlett***

Department of Pharmaceutical and Biomedical Sciences  
 University of Georgia  
 Athens, GA  
 USA

***Babak Basiri***

Department of Pharmaceutical and Biomedical Sciences  
 University of Georgia  
 Athens, GA  
 USA

***Raymond F. Biondolillo***

Q2 Solutions  
 Ithaca, NY  
 USA

***Ian A. Blair***

Center of Excellence in Environmental Toxicology and  
 Penn SRP Center, Perelman School of Medicine  
 University of Pennsylvania  
 Philadelphia, PA  
 USA

***John E. Buckholz***

Q2 Solutions  
 Ithaca, NY  
 USA

***Jonathan Crowther***

Ortho Clinical Diagnostics  
 Material and Process Services  
 Raritan, NJ  
 USA

***Marc De Meulder***

Pharmacokinetics-Dynamics and Metabolism,  
 Regulated Development Bioanalysis  
 Janssen Research & Development, a Division of Janssen  
 Pharmaceutica NV  
 Beerse  
 Belgium

***Yuzhong Deng***

Drug Metabolism and Pharmacokinetics  
 Genentech  
 South San Francisco, CA  
 USA

***Lieve Dillen***

Pharmacokinetics-Dynamics and Metabolism,  
 Regulated Development Bioanalysis  
 Janssen Research & Development, a Division of Janssen  
 Pharmaceutica NV  
 Beerse  
 Belgium

***John C. Fanaras***

Nucro-Technics  
 Scarborough, Ontario  
 Canada

***Markus Fischer***

University of Hamburg, Hamburg School  
 of Food Science  
 Hamburg  
 Germany

***Hong Gao***

Drug Metabolism & Pharmacokinetics  
 Vertex Pharmaceuticals Incorporated  
 Boston, MA  
 USA

***Lili Guo***

Center of Excellence in Environmental Toxicology and  
Penn SRP Center, Perelman School of Medicine  
University of Pennsylvania  
Philadelphia, PA  
USA

***Joerg Heeren***

University Medical Center Hamburg-Eppendorf (UKE),  
Institute of Biochemistry and Molecular Cell Biology  
Hamburg  
Germany

***Mike (Qingtao) Huang***

Clinical Pharmacology  
Akros Pharma Inc.  
Princeton, NJ  
USA

***Qin C. Ji***

Bioanalytical Sciences  
Bristol-Myers Squibb Co.  
Princeton, NJ  
USA

***Wenying Jian***

Bioanalytical & Pharmacokinetics  
Janssen Research & Development, LLC  
Spring House, PA  
USA

***Clara John***

University Medical Center Hamburg-Eppendorf (UKE),  
Institute of Biochemistry and Molecular Cell Biology  
Hamburg  
Germany

***Barry R. Jones***

Q2 Solutions  
Ithaca, NY  
USA

***Shane M. Lamos***

Department of Chemistry  
Saint Michael's College  
Colchester, VT  
USA

***Ning Li***

Department of Pharmaceutical Analysis, School of  
Pharmacy  
Shenyang Pharmaceutical University  
Shenyang  
China

***Shuwei Li***

Institute for Bioscience and Biotechnology Research  
University of Maryland College Park  
Rockville, MD  
USA

***Xiaorong Liang***

Drug Metabolism and Pharmacokinetics  
Genentech  
South San Francisco, CA  
USA

***Zhongping (John) Lin***

Bioanalytical and Biologics Services  
Frontage Laboratories  
Exton, PA  
USA

***Guowen Liu***

DMPK, Agios Pharmaceuticals  
Cambridge, MA  
USA

***Clementina Mesaros***

Penn SRP and Center for Excellence in Environmental  
Toxicology, Department of Systems Pharmacology and  
Translational Therapeutics  
University of Pennsylvania  
Philadelphia, PA  
USA

***Jeffrey D. Miller***

Sciex  
Framingham, MA  
USA

***Yongle Pang***

Department of Chemistry  
Michigan State University  
East Lansing, MI  
USA

***Shefali Patel***

Bioanalytical & Pharmacokinetics  
Janssen Research & Development, LLC  
Spring House, PA  
USA

***Jun Qu***

Department of Pharmaceutical Sciences  
School of Pharmacy and Pharmaceutical Sciences  
Buffalo, NY  
USA and

New York State Center of Excellence in Bioinformatics  
and Life Sciences  
Buffalo, NY  
USA

**Ashkan Salamatipour**

Penn SRP and Center for Excellence in Environmental Toxicology, Department of Systems Pharmacology and Translational Therapeutics  
University of Pennsylvania  
Philadelphia, PA  
USA

**Shichen Shen**

Department of Biochemistry  
Jacobs School of Medicine and Biomedical Sciences,  
University at Buffalo, State University of New York  
Buffalo, NY  
USA and  
New York State Center of Excellence in Bioinformatics  
and Life Sciences  
Buffalo, NY  
USA

**Chuan Shi**

Case Western Reserve University  
Cleveland, OH  
USA

**Nathaniel Snyder**

A.J. Drexel Autism Institute  
Drexel University  
Philadelphia, PA  
USA

**Daniel Tamae**

Department of Systems Pharmacology and  
Translational Therapeutics  
Center of Excellence in Environmental Toxicology,  
Perelman School of Medicine, University of  
Pennsylvania  
Philadelphia, PA  
USA and  
Current address: Department of Chemistry and  
Biochemistry  
California State University  
Northridge, CA  
USA

**Aimin Tan**

Bioanalytical Laboratory  
Nucro-Technics  
Scarborough, Ontario  
Canada

**Stefani N. Thomas**

Department of Pathology, School of Medicine  
Johns Hopkins University  
Baltimore, MD  
USA

**Tom Verhaeghe**

Pharmacokinetics-Dynamics and Metabolism,  
Regulated Development Bioanalysis  
Janssen Research & Development, a Division of Janssen  
Pharmaceutica NV  
Beerse  
Belgium

**QingQing Wang**

Center of Excellence in Environmental  
Toxicology and Penn SRP Center, Perelman  
School of Medicine  
University of Pennsylvania  
Philadelphia, PA  
USA

**Cong Wei**

Drug Metabolism & Pharmacokinetics  
Vertex Pharmaceuticals Incorporated  
Boston, MA  
USA

**Naidong Weng**

Bioanalytical & Pharmacokinetics  
Janssen Research & Development, LLC  
Spring House, PA  
USA

**Philipp Werner**

University of Hamburg, Hamburg  
School of Food Science  
Hamburg  
Germany

**Katrina E. Wiesner**

Department of Chemistry  
Saint Michael's College  
Colchester, VT  
USA

**Yuan-Qing Xia**

Sciex  
Framingham, MA  
USA

**Shuang Yang**

Department of Pathology, School  
of Medicine  
Johns Hopkins University

Baltimore, MD  
USA and  
Current address: Center for Biologics  
Evaluation and Research, Office of Vaccine  
Research and Review, Laboratory of Bacterial  
Polysaccharides Vaccine Structure Group  
FDA  
Ellicott City, MD  
USA

**Long Yuan**  
Bioanalytical Sciences  
Bristol-Myers Squibb Co.  
Princeton, NJ  
USA

**Guodong Zhang**  
Bioanalytical and Biomarker Development  
Shire  
Lexington, MA  
USA

**Stanley (Weihua) Zhang**  
Ortho Clinical Diagnostics  
Material and Process Services  
Raritan, NJ  
USA and  
Current address: Merck Manufacturing Division  
Global Vaccine & Biologics Commercialization  
West Point, PA  
USA

## Preface

Biomarker has been increasingly playing a significant role in drug discovery and development. Its application ranges from target and candidate selection and refinement in discovery to safety and efficacy evaluation in drug development and to patient stratification and market differentiation at late phase development and post market. With the assistance of ever-improving mass spectrometry in conjunction with liquid chromatography, assays for new and novel biomarkers, some of which at very low levels, are being developed and validated for being applied in abovementioned areas. At mean time, regulatory bodies such as the FDA have increased their drug approval evaluation using information driven by biomarkers. Yet, assay establishment for biomarkers remains to be a daunting task, partially due to the fact that biomarkers inherently are endogenous analytes, and therefore the existing assay validation guidance, albeit very useful for drug candidates, is less straightforward for the biomarkers, partially due to the lack of thorough understanding of the relationship between a target biomarker and its usage in decision making.

A comprehensive bioanalytical overview on quantitative liquid chromatography with mass spectrometric detection (LC–MS) analysis of biomarkers appropriate to the drug discovery/development stage and usage of biomarkers as discussed in this book is timely needed in the industry. For a given biomarker that can play pivotal role in drug discovery and development go-no-go decision, the bioanalytical assay needs to be appropriately established to meet both the regulatory and scientific rigor. It is the hope that this book will illustrate this concept using real-life examples.

This book contains 26 chapters that are divided into three parts. Part 1 (Chapters 1–7) provides an overview of quantitative bioanalysis of biomarkers using LC–MS. Chapter 1 provides an overall introduction of targeted quantitation of biomarkers and its application, and Chapter 2 describes the role of biomarker in drug discovery and development, with emphasis on clinical application. In Chapter 3, a thorough review of current regulatory landscape on biomarker quantitation and

industrial practices on biomarker method validation/qualification strategy is discussed. Important considerations in biomarker analysis method development and assay establishment are discussed in detail in Chapters 4–6. Chapter 4 introduces modern LC–MS on bioanalysis with emphasis on state-of-art mass spectrometry and liquid chromatography for targeted biomarker quantitation. Chapter 5 compares the advantages and disadvantages of LC–MS-based biomarker quantitation with traditional ligand binding assays. Sample preparation is probably the most important parameter for a successful biomarker assay establishment. Review and contrast of the sample preparation strategies and challenges for biomarkers and the different sample preparation procedures for various types of biomarkers (small molecules, peptides, and proteins) are the subject of Chapter 6. Several practical and successful approaches are discussed in Chapter 7, in particular, the surrogate matrix versus surrogate analyte approaches. Preanalytical consideration is pivotally important for the successful biomarker assay establishment.

Part 2 (Chapters 8–12) presents the challenges of bioanalysis quantitation and approaches to overcome those challenges. As biomarkers are endogenous compounds, they present unique challenges to appropriately establish assay parameters that are usually prescribed to drug candidates. Study design to maximize the opportunity to observe biomarker changes including sample collection, stability, circadian rhythm, etc. is extensively discussed in Chapter 8. Biomarker analyte loss due to nonspecific adsorption to the container is a frequently observed phenomenon, and Chapter 9 is specifically designed to address this issue. Another fundamental challenge for biomarker measurement is the lack of assay sensitivity. Strategies for improving sensitivity using novel strategy such as immunoaffinity extraction, derivatization, etc. are the subjects for Chapter 10. Strategy to address assay specificity, fundamentally a difficult task due to the endogenous nature of the biomarkers, is covered in Chapter 11. One of the aims of this book is also to leverage technologies that are already used in other

disciplines such as proteomics. Chapter 12 certainly bridges this by presenting novel quantitative strategy of using heavy and light labeled derivatives for the relative quantitation of biomarkers.

Part 3 (Chapters 13–26) focuses on in-depth discussion of different types of biomarkers and demonstrates case studies for their targeted quantitation using LC–MS. The first four chapters focus on biomarkers related to amino acids, peptides, proteins, and glycoproteins (Chapters 13–16). Quantitative measurement of amino acids in biological matrices can yield important information into disease identification and monitoring, treatment efficacy, and overall improvement of human health. Chapter 13 offers guidance to ensure optimal assay characteristics such as accuracy, precision, and selectivity for LC–MS quantitation of amino acid biomarkers. In Chapter 14, targeted quantification of peptide biomarkers and a case study of amyloid peptides are presented. Aspects on important issues related to biomarker assays such as adsorption, solubility, and stability are discussed in detail. Chapter 15 focuses on proteins, one of the most important types of biomarkers, and analytical approaches using signature peptide following enzymatic digestion of the proteins (bottom-up) and direct analysis of whole (intact) proteins without enzymatic digestion (top-down) are the subjects of this chapter. Key aspects in assay establishment such as sample preparation, use of internal standards, assay sensitivity and throughput, etc. are also discussed. Chapter 16 reviews recent technological advances related to glycoproteomics and glycomics biomarker analyses. Glycosylation is one of the most common protein modifications that could alter protein function and lead to various physiological and pathological consequences.

Part 3 continues with review and case studies of another class of important biomarkers related to lipid structures such as lipids (Chapter 17), hormones (Chapter 18), sterols (Chapter 19), bile acids (Chapter 20), and vitamins (Chapter 21). Chapter 17 focuses on targeted LC–MS methods for measuring class I lipids—the fatty acyls in biological samples—and the various methodologies presented in this chapter demonstrate the recent advancement in the field of lipidomics and allow for more efficient quantitation and monitoring of lipid metabolites. Chapter 18 describes the targeted LC–MS quantification of androgens and estrogens for biomarker development for hormone-dependent tumors such as that of the breast and prostate. Chapter 19 mainly focuses on the current discussions of some of the glucocorticoids and sterols as biomarkers and their corresponding bioanalysis by LC–MS, in particular 4 $\beta$ -hydroxycholesterol, a potential P450 3A4/5 endogenous biomarker. Since bile acids play a role for the development and for the therapy

of certain metabolic diseases, Chapter 20 discusses the importance of having access to an appropriate analytical platform to adequately quantify cholesterol and bile acids species. Chapter 21 reviews vitamins as biomarkers for vitamin status and deficiency. It focuses on vitamin D, not only because its deficiency is a worldwide problem but also because that there are multiple competing quantification methodologies besides LC–MS. The diversity in assay methods as well as high variability in measurements has caused controversy and confusion in clinical testing.

Part 3 concludes with review and case studies of other important biomarkers with diversified structures such as acyl-coenzyme A thioesters (Chapter 22), neurotransmitters (Chapter 23), carbohydrates (Chapter 24), nucleotides/nucleosides (Chapter 25), and oligonucleotides (Chapter 26). Chapter 22 discusses the structure and function of acyl-coenzyme A thioesters, provides an overview of the LC–MS-based methods of quantifying acyl-coenzyme A thioesters, and gives specific examples of the analysis of acyl-coenzyme A thioesters as biomarkers for current drug targets, metabolic diseases, and drug metabolism. Chapter 23 discusses the recent LC–MS methodologies developed for the measurement of neurotransmitters, which combine optimized sample preparation, chemical derivatization, and chromatographic conditions. They enable more sensitive and specific measurement of neurotransmitters in low concentration ranges, with reproducibility, high throughput, and short run time. Chapter 24 discusses the critical role of targeted LC–MS methods for quantitative analysis of carbohydrates from biological fluids. Optimal assay conditions require careful consideration of sample extraction, chromatography, and mass spectrometric detection. Chapter 25 is an informative source for LC–MS assays for the measurement of nucleoside/nucleotide biomarkers in biological samples and how to overcome challenges related to the determination of nucleosides/nucleotides due to their low abundance, high polarity, and serious matrix interferences. Chapter 26 focuses on LC–MS of oligonucleotides, which is highly challenging. Due to the highly polar nature of oligonucleotides, ion pair chromatography is typically used to enhance retention time. Various sample preparation methods may be tried to select the optimal condition.

We believe that our mission of providing a practical and bioanalytical focused book on LC–MS quantitation of endogenous biomarkers is accomplished. This book demonstrates practically how LC–MS analysis for biomarkers should be executed with great consideration of important assay parameters ranging from sample collection to assay qualification, stability assessment,

and sensitivity and specificity. With sound scientific and regulatory knowledge, one can confidently establish assays suitable for the purpose of the study. This book is only possible because of the commitment and diligence of all the authors of the book chapters. We would like to sincerely acknowledge them for their dedication and sacrifice. Kudos also go to our colleagues from Pharmacokinetics, Dynamics and Metabolism at Janssen Research & Development for their generous support and enthusiastic discussion of various topics in this book. We would also like to thank the family members

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Naidong Weng, Ph.D.  
Wenying Jian, Ph.D.

## Abbreviations

1,5-AG	1,5-Anhydro-D-glucitol
24S-OHC	24S-Hydroxycholesterol
27-OHC	27-Hydroxycholesterol
2D-LC	Two-dimensional liquid chromatography
2HG	2-Hydroxyglutarate
4 $\alpha$ -OHC	4 $\alpha$ -Hydroxycholesterol
4 $\beta$ -OHC	4 $\beta$ -Hydroxycholesterol
6 $\beta$ -HC	6 $\beta$ -Hydroxycortisol
AA	Abiraterone acetate
AA	Ascorbic acid
AA	Arachidonic acid
AAPS	American Association of Pharmaceutical Scientists
ACC	Acetyl-CoA carboxylase
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACR	Acute cellular rejection
AD	Alzheimer's disease
ADMA	Asymmetric dimethylarginine
ADME	Absorption, distribution, metabolism, and excretion
ADP	Adenosine-5'-diphosphate
AEs	Adverse effects
AIDS	Immunodeficiency diseases
AKI	Acute kidney injury
ALT	Alanine transaminase
AMI	Acute myocardial infarction
AML	Acute myeloid leukemia
AMP	Adenosine-5'-monophosphate
APCI	Atmospheric pressure chemical ionization
APP	Amyloid precursor protein
APPI	Atmospheric pressure photoionization
AQL	Above quantitation limit
AQUA	Absolute quantification
ARDS	Acute respiratory distress syndrome
ARG-1	Arginase
AST	Aspartate transaminase
ATP	Adenosine-5'-triphosphate
BA	Bile acid
BA	Benzoic acid
BAAT	Bile acid-CoA:amino acid <i>N</i> -acyltransferase
BCAAs	Branched-chain amino acids (valine, leucine, and isoleucine)
BDNF	Brain-derived neurotrophic factor

BE	Bioequivalence
BLAs	Biologics license applications
BNP	Brain natriuretic peptide
BQP	Biomarker Qualification Program
BQRT	Biomarker Qualification Review Team
BSA	Bovine serum albumin
BTA	Bladder tumor-associated antigen
BUN	Blood urea nitrogen
CAA	Cancer-associated antigens
cAMP	Cyclic adenosine-3',5'-monophosphate
CBA	Conjugated bile acid
CBQC	COPD Biomarkers Qualification Consortium
CDMS	Clinically definite multiple sclerosis
CDx	Companion diagnostics
CDX2	Caudal-type homeobox transcription factor 2
CE	Capillary electrophoresis
CE	Collision energy
CEA	Carcinoembryonic antigen
CEC	Capillary electrochromatography
CF	Cystic fibrosis
cGMP	Cyclic guanosine-3',5'-monophosphate
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHI3L1	Chitinase-3-like protein 1
CI	Chemical ionization
CI	Calcium ionophore
cICAT	Cleavable ICAT
CID	Collision-induced dissociation
CIS	Clinically isolated syndrome
CKD	Chronic kidney disease
CKs	Cytokeratins
CLIA	Clinical Laboratory Improvement Amendments
CLIA	Chemiluminescent immunoassay
CMS	Centers for Medicare and Medicaid Services
CNS	Central nervous system
CoA	Coenzyme A
COPD	Chronic obstructive pulmonary disease
COU	Context-of-use
COXs	Cyclooxygenases
CPI	Critical path initiative
CRC	Colorectal cancer
CROs	Contract research organizations
CRP	C-reactive protein
CSC	Cell surface capturing
CSF	Cerebrospinal fluid
CSP	Chiral stationary phase
CTC	Circulating tumor cell
CTLA-4	Cytotoxic T lymphocyte antigen-4
cTnI	Cardiac troponins I
cTnT	Cardiac troponins T
CTP	Cytidine-5'-triphosphate
CV	Coefficients of variation
CVD	Cardiovascular disease
CYP	Cytochrome
CZE	Capillary zone electrophoresis

D-2HG	D-enantiomer of 2-hydroxyglutarate
DA	Dopamine
DART	Direct analysis in real time
DBEMM	Dibenzyl ethoxymethylene malonate
DBS	Dried blood spot
DDI	Drug–drug interaction
DDTs	Drug development tools
DEEMM	Diethyl ethoxymethylenemalonate
DEQAS	Vitamin D External Quality Assessment Scheme
DESI	Desorption electrospray ionization
DEX	Dexamethasone
DHA	Docosahexaenoic acid
DHAA	Dehydroascorbic acid
DHEA	Dehydroepiandrosterone
DHETs	Dihydroxyicosatrienoic acids
DHT	5 $\alpha$ -dihydrotestosterone
DIART	Deuterium isobaric amine reactive tag
DMF	Dimethylformamide
DMN	Dimethylnitrosamine
DMS	Differential mobility spectrometry
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTIMS	Drift-time ion-mobility spectrometry
DTT	Dithiothreitol
E <sub>1</sub>	Estrone
E <sub>2</sub>	Estradiol
EBF	European Bioanalytical Forum
ECAPCI-MS	Electron capture atmospheric pressure chemical ionization mass spectrometry
ECD	Electron-capture dissociation
EDCs	Endocrine-disrupting chemicals
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
EETs	Epoxyicosatrienoic acids
EGFR	Epidermal growth factor receptor
EI	Electron ionization
EIC	Extracted ion chromatogram
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ENCODE	Encyclopedia of DNA Elements
EpCAM	Epithelial cell adhesion molecule
EPI	Enhanced product ion spectra
ERHIC	Electrostatic repulsion hydrophilic interaction chromatography
ESI	Electrospray ionization
ESR1	Estrogen receptor
ETD	Electron-transfer dissociation
FA	Friedreich's ataxia
FA	Fatty acid
FA	Fatty acyls
FA	Formic acid
FAIMS	Field-asymmetric waveform ion-mobility spectrometry
FAS	Fatty acid synthase
FASP	Filter-aided sample preparation
FDA	Food and Drug Administration
FEV <sub>1</sub>	Forced expiratory volume in 1 s

FFPET	Formalin-fixed, paraffin-embedded tissue
FIA	Flow injection analysis
FIA	Fluorescent immunoassay
Fmoc-Cl	9-Fluorenylmethoxycarbonyl chloride
FTICR	Fourier transform ion cyclotron resonance
FXR	Farnesoid X receptor
G3DH	Glucose-3-dehydroxidase
GABA	Gamma-amino butyric acid
GAGs	Glycosaminoglycans
GALNS	<i>N</i> -acetylgalactosamine-6-sulfatase
GBC	Global Bioanalytical Consortium
GBM	Glioblastoma multiforme
GCC	Global CRO Council for Bioanalysis
GCCs	Glucocorticoids
GC-MS	Gas chromatography in conjunction with MS
GDF-8	Growth and differentiation factor 8
GFR	Glomerular filtration rate
GIG	Glycoprotein immobilization for glycan extraction
GIP	Insulinotropic polypeptide
GirP	Girard's reagent P
GirT	Girard's reagent T
GlcHb	Glycated hemoglobin
GLDH	Glutamate dehydrogenase
GLP	Good laboratory practices
GLP-1	Glucagon-like peptide 1
GP-IS	Guanidinated protein as analog IS
gRNA	Guide RNAs
GSH	Glutathione
GST	Glutathione- <i>S</i> -transferase
GTP	Guanosine-5'-triphosphate
HA	Histamine
HbA1c	Hemoglobin A1c
HCD	Higher energy collisional dissociation
HDoHE	DHA hydroxide
HER2	Human epidermal growth factor receptor 2
HETEs	Hydroxyeicosatetraenoic acids
HGF	Hepatocyte growth factor
HIC	Hydrophobic interaction chromatography
HILIC	Hydrophilic interaction chromatography
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxymethyl-3-glutaryl-CoA
HPAA	Hypothalamus–pituitary–adrenal axis
HpDoHE	Hydroperoxide
HPETEs	Hydroperoxyeicosatetraenoic acids
HRMS	High-resolution MS
IA	Invasive aspergillosis
IA	Immunoassay
IAE	Immunoaffinity extraction
IBCF	Isobutyl chloroformate
ICATs	Isotope-coded affinity tags
ICPL	Isotope-coded protein label
IDA	Information-dependent acquisition
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing

IgG	Immunoglobulin G
IGOT	Isotope-coded glycosylation-site-specific tagging
IHC	Immunohistochemistry
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-21	Interleukin 21
IL-18	Interleukin 18
IMAC	Immobilized metal affinity chromatography
IMER	Immobilized enzymatic reactor
IMS	Ion mobility MS
IND	Investigational new drug
iPSC	Induced pluripotent stem cells
IQ	Innovation and quality
IS	Internal standard
IsoTaG	Isotope-targeted glycoproteomics
iTRAQ	Isobaric tags for relative and absolute quantification
IUPAC	International Union of Pure and Applied Chemistry
IVD	<i>In vitro</i> diagnostic
KIM-1	Kidney injury molecule
LBA	Ligand-binding assay
LC–MS	Liquid chromatography in conjunction with mass spectrometric detection
LDH	Lactate dehydrogenase
L-DOPA	Levodopa
LDTs	Laboratory-developed tests
LIMS	Laboratory information management system
lincRNA	Long intervening noncoding RNAs
LLE	Liquid–liquid extraction
LLME	Liquid–liquid microextraction
LLOQ	Low limit of quantitation
LMAN2	Lectin mannose binding 2
lncRNAs	Long noncoding RNAs
LOD	Limit of detection
LOI	Letter of intention
LOQ	Limit of quantification
LOXs	Lipoxygenases
Lp-PLA2	Lipoprotein-associated phospholipase A2
LTs	Leukotrienes
LVEF	Left ventricular ejection fraction
LXR	Liver X receptor
MALDI–MSI	Matrix-assisted laser desorption ionization mass spectrometry imaging
MAP	Microtubule-associated protein
MBDD	Model-based drug development
MDH	Malate dehydrogenase
MEPS	Microextraction by packed sorbent
MGMT	$O^6$ -methylguanine DNA-methyltransferase
MHPG	3-Methoxy-4-hydroxyphenylethylene glycol
miRNAs	MicroRNAs
MMA	Mono-methylarginine
MMP9	Matrix metallopeptidase 9
MoAs	Mechanism of actions
MPA	Metaphosphoric acid
MPO	Myeloperoxidase
MPS	Mucopolysaccharidosis
MR	Metabolic ratio

MRI	Magnetic resonance imaging
MRM	Multiple reaction mode
mRNA	Messenger RNA
MS	Mass spectrometry
MSI	MS imaging
MSIA	Mass spectrometric immunoassay
MUGA	Radionuclide ventriculogram
NaClO	Sodium hypochlorite
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide
NADP <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate
NAEs	<i>N</i> -acylethanolamines
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NCDs	Non-communicable diseases
ncRNA	Noncoding RNAs
NDAs	New drug applications
NGAL	Neutrophil gelatinase-associated lipocalin
NGF	Nerve growth factor
NIH	National Institutes of Health
NIST	National Institute of Standards and Technology
NMP22	Nuclear matrix protein
NO	Nitric oxide
NOS	Nitric oxide synthase
NPLC	Normal-phase liquid chromatography
NSAIDs	Nonsteroidal anti-inflammatory drugs
NSB	Nonspecific binding
NSCLC	Non-small cell lung cancer
NSTEMI	Non ST-segment elevation myocardial infarction
NTCP	Sodium-dependent sodium-taurocholate cotransport polypeptide
NTs	Neurotransmitters
nUHPLC	Nanoflow UHPLC
NUMA1	Nuclear mitotic apparatus protein
OATP	Organic anion transporting proteins
OCBs	Oligoclonal bands
ORNG	Oxidative release of natural glycans
OXM	Oxyntomodulin
PAD	Pulsed amperometric detector
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PD	Parkinson's disease
PD-1	Programmed death-1
PET	Positron emission tomography
PFB	Pentafluorobenzyl
PFP	Pentafluorophenyl
PGD <sub>2</sub>	Prostaglandin D2
PGE <sub>2</sub>	Prostaglandin E2
PGF <sub>2</sub> α	Prostaglandin F <sub>2</sub> α
PGR	Progesterone receptor
PGs	Prostaglandins
piRNAs	piwi-interacting RNAs
PK/PD	Pharmacokinetics/pharmacodynamics

PKU	Phenylketonuria
PLE	Pressurized liquid extraction
PMA	Premarket approval application
POC	Point of care
PPT	Protein precipitation
PRA	Plasma renin activity
PRM	Parallel reaction monitoring
PSA	Prostate-specific antigen
PSA-ACT	PSA bound to $\alpha$ 1-antichymotrypsin
pSILAC	Pulsed SILAC
PTGS	Prostaglandin-endoperoxide synthase
PTMs	Posttranslational modifications
PUFAs	Polyunsaturated fatty acids
QconCat	Quantification concatamer
QC <sub>s</sub>	Quality control samples
qPCR	Quantitative polymerase chain reaction
QUEST	Quantitation using enhanced signal tags
QWBA	Quantitative whole-body autoradiography
RA	Rheumatoid arthritis
RAAS	Renin–angiotensin–aldosterone system
RAM	Restricted-access material
RBC	Red blood cell
RCTs	Randomized clinical trials
RF	Response factor
rhTRAIL	Recombinant human TNF-related apoptosis inducing ligand
RIAs	Radioimmunoassays
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RO	Receptor occupancy
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RPLC	Reversed-phase liquid chromatography
RT-PCR	Reverse transcription-polymerase chain reaction
S/N	Signal/noise ratio
SALLE	Salt-assisted liquid–liquid extraction
SBSE	Stir bar sorptive extraction
SBT	Small bowel transplants
SCC	Squamous cell carcinoma
SCID	Severe combined immunodeficiency diseases
Scr	Serum creatinine
SCX	Strong cation exchange
SDBS	Sodium dodecylbenzenesulfonate
SDMA	Symmetric dimethylarginine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SERD	Selective estrogen receptor degrader
SFTI	Sunflower trypsin inhibitor
SID	Stable isotope dilution
SIL	Stable isotope labeled
SILAC	Stable isotope labeling with amino acids in cell culture
SILAP	Stable isotope labeling proteomics
SIMS	Secondary ion mass spectrometry
siRNAs	Small interfering RNAs
SISCAPA	Stable isotope standards and capture by anti-peptide antibodies

SLE	Solid-supported liquid extraction
snoRNA	Small nucleolar RNAs
SNPs	Single nucleotide polymorphisms
SOD	Surfactant-aided precipitation/on-pellet digestion
SOPs	Standard of operations
SPD	Selective peptide derivatization
SP-D	Surfactant protein D
SPE	Solid-phase extraction
SPECT	Single-photon emission computed tomography
SPEG	Solid-phase extraction of glycopeptides
SPME	Solid-phase microextraction
SPs	Signature peptides (surrogate peptides)
SRM	Selected reaction monitoring
sTREM-1	Soluble triggering receptor expressed on myeloid cells 1
SULTs	Sulfotransferases
suPAR	Soluble urokinase-type plasminogen activator receptor
SWATH	Sequential window acquisition of all theoretical
T	Testosterone
T2DM	Type 2 diabetes
TAPS	Total androgen pathway suppression
TBV	Total blood volume
TCA	Tricarboxylic acid cycle
TCA	Taurocholic acid
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine
TFE	Trifluoroethanol
TIMP-1	Tissue inhibitor of matrix metalloproteinase-1
TKV	Total kidney volume
TLC	Thin-layer chromatography
TM	Translational medicine
TMS	Trimethylsilyl
TMT	Tandem mass tag
TOF	Time of flight
TOSIL	Tandem <sup>18</sup> O stable isotope labeling
TP63	p63 Protein
T <sub>regs</sub>	Regulatory T cells
Tris	Tris(hydroxymethyl)aminomethane
TRLs	Triglyceride (TG)-rich lipoproteins
TWIMS	Traveling-wave ion mobility spectrometry
TXs	Thromboxanes
UBA	Unconjugated bile acid
UGTs	Uridine diphosphate glucuronosyltransferases
UPLC-MS/MS	Ultra-performance liquid chromatography–tandem mass spectrometry
UTP	Uridine-5'-triphosphate
VDBP	Vitamin D-binding protein
VEGF	Vascular endothelial growth factor
VMA	Vanillylmandelic acid (3-Methoxy-4-hydroxymandelic acid)
VPA	Valproic acid
WRIB	Workshop on recent issues in bioanalysis

## **Part I**

### **Overview**

## 1

## Overview of Targeted Quantitation of Biomarkers and Its Applications

Naidong Weng

*Bioanalytical & Pharmacokinetics, Janssen Research & Development, LLC, Spring House, PA, USA*

### 1.1 Introduction

In the last two decades, utilization of biomarkers in drug discovery and development has seen rapid growth as a result of the advancement of laboratory techniques and bioanalytical assays including ligand-binding assays (LBA) such as enzyme-linked immunosorbent assay (ELISA), quantitative polymerase chain reaction (qPCR), and mass spectrometry (MS)-based technologies, and so on (Anderson and Kodukula, 2014). Currently, pharmaceutical companies and regulatory authorities are actively engaged in developing robust efficacy and safety biomarkers that can be used in a translational manner to assist drug development by making the right choice for “go” and “no-go” decisions at the earliest possible stage. In order to most efficiently utilize the resources and maximize the benefits of biomarker research, most of the drug companies have established internal biomarker research centers and are also pursuing extensive collaborations with academia, hospitals, and research institutes. A biomarker can assist target and candidate selection in drug discovery, toxicity assessment, dose selection, and pharmacokinetics (PK)/pharmacodynamics (PD) modeling in drug development. In clinical Phase I–IV, a biomarker can help in patient stratification, drug–drug interaction (DDI) evaluation, efficacy assessment, safety monitoring, and companion diagnosis as well as postapproval surveillance. Biomarkers measured in patients before treatment can also be used to select patients for inclusion in a clinical trial. Changes in biomarkers following treatment may predict or identify safety problems related to a candidate drug or reveal a pharmacological activity that is expected to predict an eventual benefit from treatment. Biomarkers can also be used as diagnostic tools for the identification of population with an underlying disease and its progressive stage.

In fact, most of the drug programs in development stages have requirements of biomarkers to be incorporated in the preclinical and clinical development strategy as they can help ensure safety and efficacy of the drug candidates. Indeed, it has been reported that the ability of biomarkers to improve treatment and reduce healthcare costs is potentially greater than in any other area of current medical research (Drucker and Krapfenbauer, 2013). A search of one major clinical trial registry on December 5, 2015 (<https://ClinicalTrials.gov>), using the search term “biomarker,” generated 17,366 results, almost twofold increases, compared to what had been previously reported 5 years ago (Boulton and Dally, 2010). Less than a year later (November 8, 2016), this number is 19,611.

More specifically, biomarkers have demonstrated the added values to every major disease area. For example, in oncology, with the growth in numbers of targeted therapies for oncology clinical testing, biomarkers are often used to select patient population (Arteaga, 2003). Biomarkers can also allow investigators to stratify patients for prospective or retrospective evaluation of different clinical responses and for identification of specific responder sub-population (Mendelsohn and Baselga, 2003). A previous publication also proposed optimizing oncology drug development by using a tiered set of clinical biomarkers that predict compound efficacy with increasing confidences as well as increasing rigor of validation at each of the three levels (Floyd and McShane, 2004). Level-1 biomarkers confirm biochemical or pharmacological mechanism of action by showing that the drug is modulating its target and provides correlation of PD and PK, which is the exposure of the drug and its active metabolites. Level-2 biomarkers confirm that the drug is producing a desired PD effect directly related to its potential for efficacy such as altered downstream cell signaling in pathways related to target, decreased metabolic activity,

or changes in tumor vascular perfusion. Level-3 biomarkers have predictive power for a desired outcome and may be surrogate end points for *in vivo* symptoms, such as tumor size. It should be noted that even with the extensive research by many scientists over the last decades, very few biomarkers, that can be measured in the laboratory, qualify for Level-3 biomarkers. Of course, this type of categorization of biomarkers can also be applied to other disease areas. Almost all of the biomarkers discussed in this book belong to the first two levels.

For Type 2 diabetes (T2DM), it was estimated that, in 2010, 285 million people had been diagnosed with diabetes mellitus worldwide, a prevalence of 6.4% of the total population. This is predicted to increase to 439 million (7.7% of total population), and by 2030, T2DM will account for about 90% of diabetic patients worldwide (Shaw et al., 2010). Biomarker search has lead to several promising biomarkers such as Chitinase-3-like protein 1 (CHI3L1) also known as YKL-40, soluble CD36 (cluster of differentiation 36), leptin, resistin, interleukin 18 (IL-18), retinol-binding protein 4 (RBP4), and chemerin that could be indicative for the pathogenesis of insulin resistance and endothelial dysfunction in T2DM patients (Qhadijah et al., 2013). In another paper (Lyons and Basu, 2012), it was postulated that in blood, hemoglobin A1c (HbA1c) may be considered as a biomarker for the presence and severity of hyperglycemia, implying diabetes or prediabetes.

Alzheimer's disease (AD) is an irreversible, progressive brain disorder that slowly destroys memory and thinking skills, and eventually the ability to carry out the simplest tasks. In most people with AD, symptoms first appear in their mid-60s. Estimates vary, but experts suggest that more than five million Americans may have AD (<https://www.nia.nih.gov/alzheimers/publication/alzheimers-disease-fact-sheet>). There is significant interest in the development of methods to validate novel biomarkers for diagnosis of AD. Cerebrospinal fluid (CSF) levels of  $\beta$ -amyloid A $\beta$ 1–40 and A $\beta$ 1–42 peptides, total Tau protein, and phosphorylated Tau protein have diagnostic values in AD (Chintamaneni and Bhaskar, 2012). Tau protein is a highly soluble microtubule-associated protein (MAP). In humans, these proteins are found mostly in neurons compared to non-neuronal cells. Tau protein and phosphorylated Tau protein are measured by using ELISA (Herrmann et al., 1999). Liquid chromatography in conjunction with mass spectrometric detection (LC-MS)-based assays have also been published for measuring  $\beta$ -amyloid A $\beta$ 1–40 and A $\beta$ 1–42 peptides in CSF (Choi et al., 2013). A systematic review and meta-analysis of the literature on whether or not CSF total tau, phosphorylated tau, and  $\beta$ -amyloid A $\beta$ 1–42 peptide help predict progression of mild cognitive impairment to AD was conducted (Diniz et al., 2008).

## 1.2 Biomarker Definition

It is generally accepted in the pharmaceutical industry that a biological marker or a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathologic processes, or biological responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). Biomarkers are typically classified into diagnostic, prognostic, and predictive biomarkers. Biomarker definition and usage are summarized in Appendix II of the Guidance for Industry and FDA Staff (Qualification Process Working Group, 2014).

A *diagnostic* biomarker is a disease characteristic that categorizes a person by the presence or absence of a specific physiological or pathophysiological state or disease.

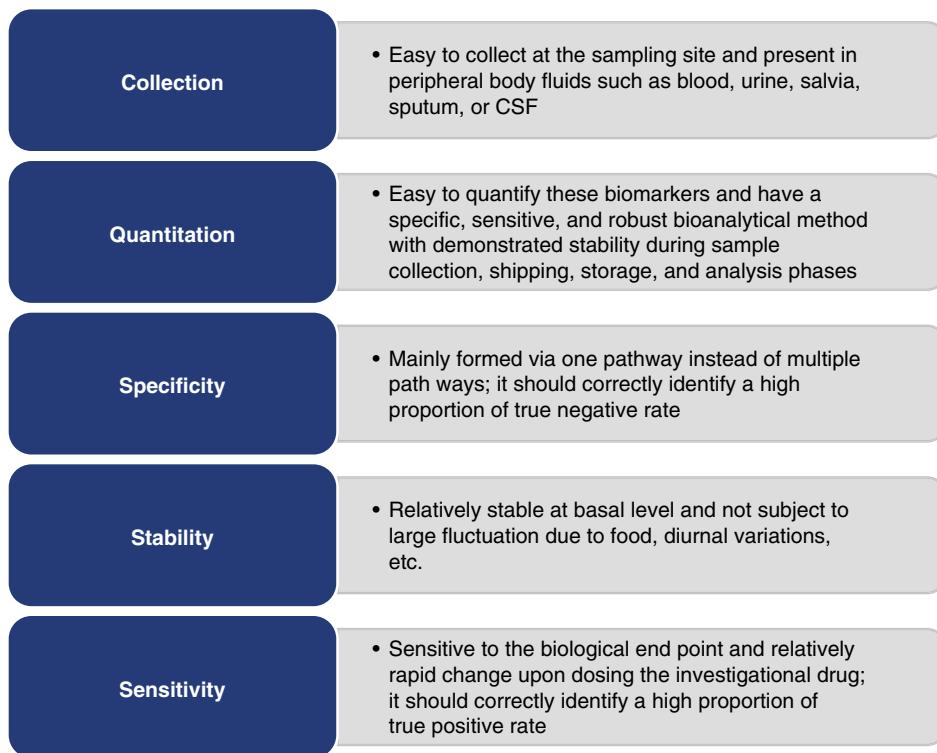
A *prognostic* biomarker is a baseline characteristic that categorizes patients by degree of risk for disease occurrence or progression of a specific aspect of a disease.

A *predictive* biomarker is a baseline characteristic that categorizes patients by their likelihood of response to a particular treatment relative to no treatment.

In pharmaceutical industry research and development (R&D), biomarkers can also be described as efficacy or safety biomarkers. Division of common biomarkers into these two categories is probably better linked with the drug discovery and development process as deficiency in safety or efficacy is the major reason for termination of drug candidates. Efficacy biomarkers emphasize on mode of action and can be used to build early confidence in drug mechanism and can potentially substitute for clinical symptoms as a measurement of efficacy. Safety biomarkers are early markers of reversible or irreversible drug-induced adverse events and can be used to understand the mechanism of drug-induced toxicity.

An emerging area of extensive research is to use endogenous biomarkers to predict potential cytochrome P450 or transporter-mediated DDI. They can be used to assess changes in drug metabolism and transport phenotype due to intrinsic and extrinsic factors. The benefits of endogenous DDI biomarkers include better PK/PD correlation due to samples collected at multiple time points; resource sparing because it is a secondary objective in a Phase I clinical study; its applicability to studies in all population; its early signal for metabolic liability without conducting a separate clinical DDI study using exogenous drugs, such as midazolam, as probes.

It is very hard to identify and validate a good DDI biomarker. This process takes a lot of research and verification as well as extensive collaboration from multiple institutes, hospitals, and consortia to confirm the initial finding. For example, bioanalysis of 4 $\beta$ -hydroxycholesterol in human plasma is currently being proposed by Innovation and Quality (IQ) consortium as a potential



**Figure 1.1** Features of ideal endogenous biomarkers on aspects of sample collection, quantitation, specificity, stability, and sensitivity.

endogenous biomarker for CYP3A4 induction (Aubry et al., 2016) even though the initial observation was made a decade ago (Bodin et al., 2001).

Ideal endogenous biomarkers including DDI biomarkers should possess the following features on sample collection, quantitation, specificity, stability, and sensitivity (Figure 1.1). Using 4 $\beta$ -hydroxycholesterol as an example, this biomarker meets essentially all of these features when used as a probe for CYP3A4 induction studies. The long half-life of 4 $\beta$ -hydroxycholesterol results in small variations in concentrations but excludes this marker in short-term studies. On the other hand, 6 $\beta$ -hydroxycortisol/cortisol ratio in urine, another frequently used endogenous biomarker for CYP3A4 inducer, is a more rapid biomarker due to short half-life with little delay time behind the changes of CYP3A4 activity in vivo. However, the short half-life and diurnal effect lead to more variable data, even with the correction in cortisol concentration (Dutreix et al., 2014).

### 1.3 Current Challenges of a Biomarker

The ultimate goal for a biomarker is the establishment of clinical utility that guides patient care, but attempts to reach this goal must be preceded by analytical and

clinical validation of the “locked-down” biomarker assay. Even though endogenous biomarkers could become a valuable tool to assess liability early in drug development, nevertheless, out of thousands of biomarkers discovered through metabolomics and proteomics approaches, only a few dozens were found to be useful in assessing efficacy and toxicity of the drug candidates. The drugs may have an impact on multiple pathways of endogenous biomarkers’ disposition and formation, and how to extrapolate a biomarker from healthy volunteers to patient population can also be a challenge (Drucker and Krapfenbauer, 2013).

Major challenges regarding integrated and harmonized processes, spanning preanalytical, analytical, and postanalytical phases of development remain (de Gramont et al., 2015). During biomarker development, robust laboratory methodology is essential at all analytical phases. Lack in biomarker characterization and validation by using robust analytical techniques, which is a lengthy process requiring careful planning and execution of assay development and validation, have been attributed to be major reasons. Problems with the collection, equipment, or transportation of specimens to the laboratory can affect the measurement of the biomarker. Improper storage of samples or changes in storage environment can also affect measurement of biomarkers (Mayeux, 2004).

## 1.4 Biomarker Validation Process

Over the last a few decades, drug discovery and development have been driven, in all therapeutic areas, by the pharmaceutical companies to become more productive and to launch more products onto the market in a cost-effective manner. Even with much investment in genomics, proteomics, metabolomics, and other “omics,” the approved rate of New Drug Applications (NDAs) remains relatively flat. Majority of compounds entering clinical trials fail and many new approved products have significant labeling restrictions. In 2004, FDA’s Critical Path Initiative (CPI) recognized that the process of drug development and the availability of new therapies were not fully benefitting from the many advances in biomedical science. In addition, drug development had become increasingly challenging and resource intensive. An important area identified by the CPI as potentially enabling significant progress in drug development was applying those scientific advances as new tools to aid the development process. Such tools could speed the availability of new products that may be safe and effective. FDA has undertaken multiple initiatives to support the development of new drug development tools (DDTs). Among these efforts has been the creation of a formal qualification process that FDA can use when working with submitters of DDTs to guide development as submitters refine the tools and rigorously evaluate them for use in the regulatory process. Because of the tremendous potential of biomarker utilization, it has been listed, along with clinical outcome assessments, and animal models, as the established qualification programs under DDT guidance.

The European Medicines Agency (EMA) also pays close attention to research in the use of biomarkers in the development of medicines. In August 2014, EMA issued a draft concept paper outlining the key elements to be developed in a guideline on good genomics biomarker practices. This is expected to facilitate the use of genomic data for the development of the so-called “personalized medicines,” the safety monitoring of medicines, and the early diagnosis of disease.

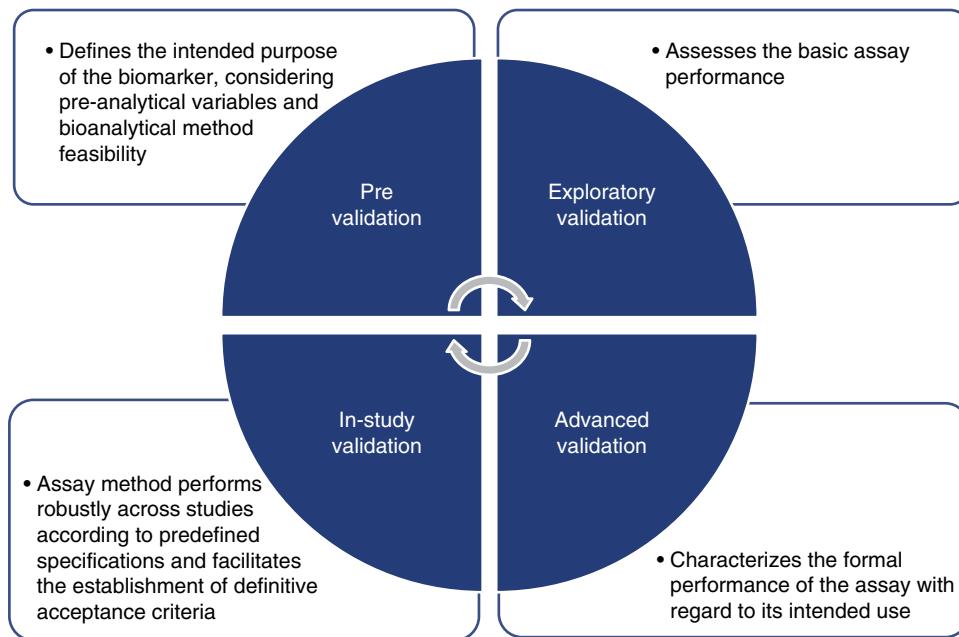
Value of biomarker measurement that can characterize baseline state, a disease process, or a response to a treatment is well recognized by both agencies. A Joint FDA/EMA Letter of Intention (LOI) submission for biomarkers and clinical outcome assessment qualification programs was issued. Parallel submissions for qualification of biomarkers to both agencies are encouraged and both FDA and EMA will share their scientific perspective, advice, and response letters for the submission. With the joint LOI, the agencies intend to reduce the time taken by applicants to prepare LOIs. A joint EMA/FDA report on kidney injury was issued and a number of biomarkers

including albumin,  $\beta$ 2-microglobulin, clusterin, cystatin C, kidney injury molecule 1 (KIM-1), total protein, and trefoil factor-3 were recommended. Discussion on biomarkers for oncology and AD were also extensively researched. For example,  $\beta$ -amyloid 42 and total Tau protein in CSF were recommended as useful biomarkers by EMA. For drug-induced cardiotoxicity biomarkers in preclinical studies, cardiac troponins T (cTnT) and I (cTnI) in serum/plasma were proposed as biomarkers. In 2015, three clinical biomarkers were proposed by FDA: Fibrinogen in plasma as a prognostic biomarker for enrichment of clinical trials in chronic obstruction pulmonary disease (COPD); an imaging biomarker measuring total kidney volume (TKV) as the prognostic biomarker for enrichment of clinical trials in autosomal dominant polycystic kidney disease; and galactomannan as a serum/bronchoalveolar lavage fluid biomarker for patient selection for enrollment in invasive aspergillosis (IA) clinical trials.

## 1.5 Current Regulatory Requirement for Target Biomarker Quantitation

Biomarkers were typically discovered via metabolomics, proteomics, system biology, or their combinations. Bioanalytical assay development and validation will be used to support retrospective pilot and prospective pilot studies. The bioanalytical assays which play pivotal roles to support all phases of biomarker validation and “lock-down” for clinical usage should have the appropriate quality to allow good, robust, and data-driven scientific decision to be made. Sensitivity analysis is then performed to find the specificity of the biomarker to the biological end point. Finally, a well-designed and controlled validation study is conducted to finally confirm the utility of the biomarker. In these two late phases, well-established bioanalytical assays play even more significant roles since the assay performance can impact not only the outcome of the sensitivity test but also the numbers of subjects and samples that have to be used to establish utility of the biomarkers. A well-established bioanalytical assay with excellent robustness and precision is essential for biomarkers, especially when its level of change from predose to postdose is small.

Currently, fit-for-purpose assay establishment was proposed for biomarker assays (Lee et al., 2006). In brief, fit-for-purpose biomarker assay validation can be separated in four continuous iterative activities (Figure 1.2). This approach was then endorsed and adapted by both the regulatory authorities (Booth, 2011; Valeri et al., 2013) and other industrial organizations such as European Bioanalytical Forum (EBF) (Timmerman et al.,



**Figure 1.2** Four continuous iterative activities of fit-for-purpose biomarker assay validation.

2012) and Global CRO Council for Bioanalysis (GCC) (Hougot et al., 2012).

In general, as drug development proceeds through the typical phases, the level of validation needed increases accordingly. There is no mention of biomarkers in the EMA bioanalytical assay validation guideline. FDA's recent inclusion of biomarkers in the 2013 draft bioanalytical method validation guidance indicates that biomarkers are used for safety, efficacy, and patient selection and treatment; therefore, the data for these compounds are as important as PK data for a new drug. Method validation should be fit-for-purpose based on the objectives of the study. The level of risk for pre-Phase I studies is different from the level of risk during Phase II; therefore, method validation requirements should be modified accordingly. When deciding how much validation is required, the analyte development platform should be considered, as well as the purpose of the assay.

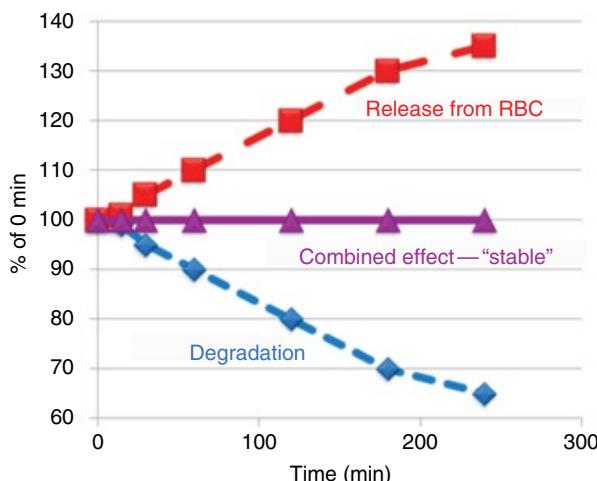
## 1.6 Challenges of Biomarker Quantitation

Biomarker quantitation can be quite challenging. One main reason is that most biomarkers are dealing with detecting diseases or toxicities in humans and animals with very low concentration level of proteins or metabolites among thousands of other proteins and metabolites. A blank biological matrix contains the analytes of interest,

which makes it difficult to find clean matrices. Circadian rhythm, food intake, and emotional state may affect the biomarker level and data interpretation (Jian et al., 2012). Specificity of the assay needs to be carefully confirmed since the *in vivo* system tends to generate multiple isomers which may interfere with quantitation. Sensitivity of detecting a very low level of biomarkers can be also problematic. Selection of assay platforms can definitely have impact on the biomarker validity. For example, when ApoA1 biomarkers were measured using LC-MS and ELISA for the same set of samples, LC-MS data indicated there was a significant difference between smokers and nonsmokers while the ELISA failed to reveal this difference (Wang et al., 2015).

Stability of biomarkers during sample collection, storage, and analysis has significant impact on the data quality and needs to be established. A lack of consistency in sample collection and storage can invalidate a study before any data can be collected. Figure 1.3 demonstrates an example that pseudostability of biomarker fatty acid amides in blood is observed due to two opposite forces: release of fatty acid amides from red blood cells and their degradation in the blood. It was only uncovered by carefully investigating the results obtained from incurred samples and from fortified quality control samples. Otherwise, a misleading sample collection procedure would be used (Jian et al., 2010).

Many biomarkers tend to stick to the surface of containers during collection, storage, and sample processing, and this nonspecific adsorption loss needs to



**Figure 1.3** Pseudostability of biomarker fatty acid amides in blood due to two opposite forces: release of fatty acid amides from red blood cells (RBC) and degradation in the blood.

be carefully examined, resolved, or mitigated. Since there is no true blank matrix, strategy of construction of calibration standards and quality control samples should also be carefully formulated, using surrogate versus authentic matrix or surrogate versus authentic analyte (Ongay et al., 2014). A surrogate matrix is prepared using artificially prepared buffer solution containing usually a small amount of proteins to mimic the authentic matrix without the presence of the biomarker. A surrogate analyte is a stable-isotope labeled analyte that can be used to construct the analytical calibration curve even below the endogenous level of the biomarkers, thus making it feasible to quantify low level endogenous biomarkers. In order to compensate for the assay variability caused by incomplete extraction recovery and ionization suppression/enhancement from matrix components, internal standard is usually used, which is fortified to the sample in a quantitative manner at the earliest step of sample processing. The use of stable isotope labeled internal standard, which has almost identical physical and chemical properties as the biomarker analytes, provides the highest analytical specificity possible for quantitative biomarker determinations. The use of appropriate protein standards in LC-MS assays is critically important and is an active area of research within the field of protein biomarkers (Ciccimaro and Blair, 2010).

In a bioanalytical laboratory, the time used for developing a robust bioanalytical method for biomarker measurement is typically two to threefolds higher than the time used for establishing bioanalytical assay for a drug candidate.

## 1.7 Current Technologies for Biomarker Quantitation

### 1.7.1 LC-MS

Analysis of biomarkers by LC-MS has seen rapid increase in the last decade. Current advances of chromatographic stationary phases and applications of LC-MS for biomarker research were reviewed in literature (Cummings et al., 2009; Denoroy et al., 2013; Chappell et al., 2014). The advantages and applications of LC-MS for biomarker analysis are well covered in the following chapters of this book. Small molecule biomarkers can be usually analyzed directly without derivatization or with derivatization to enhance their detectability (e.g., 4- $\beta$  hydroxycholesterol) (Barnaby et al., 2015; Niwa et al., 2015; Zhu et al., 2015). Aided by the highly sensitive mass spectrometry instrument, more efficient LC, and better understanding of sample preparation, it is not unusual to achieve sub ng/mL sensitivity of measurement for many types of small molecule biomarkers (Houghton et al., 2009).

On the large molecule side, significant progress is also being made to establish robust bioanalytical assays for measuring biomarkers (Berna et al., 2008; Ackermann, 2009; Palandra et al., 2013; Wang et al., 2014). However, even with rapid progress on innovative sample clean-up procedure such as immunoaffinity capture, nano- and microflow LC for more efficient ionization, and various enzymatic digestion procedures to generate surrogate peptides, current LC-MS still lags behind on sensitivity for the measurement of many protein biomarkers by ELISA and in particular RNA/DNA biomarkers by qPCR, especially on the intact level. In the foreseeable future, it can be anticipated that LC-MS will play a complementary role for the ELISA of protein biomarkers and qPCR of RNA/DNA biomarkers. It is important to note that using different analytical methods, different conclusions may be drawn, as already discussed previously for ApoA1, a potential endogenous biomarker for cardiovascular diseases. When ApoA1 was measured by LC-MS, there was a statistically significant difference between smokers and nonsmokers while the ELISA assay for the same set of samples did not indicate that (Wang et al., 2015).

### 1.7.2 GC-MS

Gas chromatography in conjunction with MS (GC-MS), which predated LC-MS, offers some unique advantages for measuring small molecule biomarkers. GC typically provides higher resolution power than LC. Excellent resolution of the biomarker of interest from its interference peaks was achieved with an extremely sharp peak (Zimmermann and Jackson, 2010). A metabolomic

study of biomarkers associated with dimethylnitrosamine (DMN)-induced hepatic fibrosis in Sprague-Dawley rats was performed using GC-MS (Ju et al., 2013). This high chromatographic resolution power can come handy when chromatographic resolution of isobaric isomers of a biomarker is needed. GC-MS can also be more sensitive than LC-MS for some biomarkers. Although most biomarkers would need a derivatization step to make them volatile and suitable for GC-MS analysis, some volatile biomarkers can be analyzed directly without derivatization.

### 1.7.3 Ligand-Binding Assay

A typical LBA utilizes an analyte-specific binder (typically antibody but may include other binders such as binding protein, drug, target protein, or receptor) to capture the analyte of interest. The captured analyte is detected by the “detector molecule” which is generally an antibody labeled with a radioisotope (e.g.,  $^{125}\text{I}$ ), an enzyme (e.g., horseradish peroxidase, alkaline phosphatase), or another label (e.g., biotin, avidin). ELISA, the most commonly used LBA, generally uses a detector molecule that is labeled with an enzyme. The extent of enzyme activity is measured by the changes in color (or fluorescence) intensity of the substrate solution. The color intensity is directly proportional to the concentration of analyte captured on the microtiter plate.

LBA is extremely sensitive and is currently still the method of choice for large molecule biomarkers such as proteins (Sloan et al., 2012). LBA also has higher throughput than LC-MS analysis. On the other hand, developing a suitable antibody can be tedious and careful control of assay parameters such as critical reagents and parallelism is very important (Stevenson, 2012; Stevenson and Purushothama, 2014). Due to the nature of indirect measurements in LBA, the results are somewhat less precise than chromatographic assays. Due to the limited analyte-binding capacity of the binder molecule (e.g., capture antibody), the typical calibration curves in these assays are nonlinear, as opposed to the linear curves in chromatographic assays. Consequently, the range of quantification in an LBA is narrower than in the linear curves of chromatographic assays. The resulting high concentration of biomarkers in the study samples may require sample dilutions. The other potential drawback of LBA is potential lack of selectivity due to cross-reactivity of the capturing antibodies with multiple compounds in the matrix.

### 1.7.4 Flow Cytometry

Microparticle-based flow cytometric assays for determination of biomarkers has gained tractions over the last decade (Wu et al., 2010). A large number of analytes can

be measured on these multiplex systems simultaneously (Vignal, 2000). The technology utilizes microspheres as the solid support for a conventional immunoassay, affinity assay, or DNA hybridization assay which are subsequently analyzed on a flow cytometer, although the initial setup can be time consuming and expensive.

### 1.7.5 Quantitative PCR (qPCR)

qPCR is a powerful and sensitive gene analysis technique and it measures PCR amplification as it occurs. Typically, a qPCR program consists of a series of 20 to 40 repeated temperature changes, called cycles, with each cycle commonly consisting of two to three discrete temperature steps, usually three (e.g., 94–96°C for denaturation, ~68°C for annealing, and 72°C for elongation). qPCR is extremely sensitive (sub pg/mL range) and has become the gold standard for measuring DNA and RNA including both drugs and biomarkers in biological fluids (Wang et al., 2013a, 2013b; Wang and Ji, 2016). A reverse transcript step is needed to convert RNA into a complementary DNA template, which is then amplified with real-time detection of fluorescence. During amplification, a fluorescent dye binds, either directly or indirectly via a labeled hybridizing probe, to the accumulating DNA molecules, and fluorescence values are recorded during each cycle of the amplification process. The fluorescence signal is directly proportional to DNA concentration over a broad range, and the linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. However, it suffers from low specificity and low accuracy/precision (up to  $\pm 50\%$ ) as well as high reagent costs.

## 1.8 Current Biomarker Quantitation Applications

Many applications of biomarker quantitation have been reported in literature. It is not possible to have a comprehensive review in this chapter. In the following chapters of this book, a more detailed discussion of various types of biomarkers is provided. Some representative examples using various technologies discussed in the previous section are illustrated here.

### 1.8.1 Protein Biomarkers

Proteins are very diverse and therefore potentially informative as biomarkers. Challenges for developing new protein-based biomarkers include the complexity of protein composition in blood, the diversity of post-translational modifications, the low relative abundance of many proteins of interest, the sequence variations among different clinically relevant species, and most

importantly, the difficulties in developing suitable high sensitivity bioanalytical assays. Discovery and development of new protein-based biomarkers with proper characteristics is an expensive and time-consuming task. ELISA has been traditionally employed for protein biomarker measurement (DeSilva et al., 2003; Lee, 2009; Valentina et al., 2011). ELISA assay is very sensitive but may suffer from cross-reactivity of similar endogenous proteins.

Acute kidney injury (AKI) has been defined as a rapid decline in glomerular filtration rate. Diagnosis of AKI is frequently based on measurements of blood urea nitrogen (BUN). BUN and serum creatinine, another commonly used biomarker for AKI, are not very specific or sensitive for the diagnosis of AKI because they are affected by many renal and nonrenal factors that are independent of kidney injury or kidney function. Urinary kidney injury molecule (KIM-1) is proposed as a sensitive quantitative biomarker for early detection of kidney tubular injury (Han et al., 2002; Mussap et al., 2014). A validated sandwich ELISA assay for measuring KIM-1 in urine was reported (Chaturvedi et al., 2009). Linearity, intra-run precision, inter-run precision, lower limit of quantification, recovery, dilution verification, reference range, stability, and length of run were established. The low limit of quantitation (LLOQ) is 59 pg/mL.

CHI3L1, also known as YKL-40, a member of family 18 glycosyl hydrolases, is secreted by cancer cells. YKL-40 was determined by ELISA in plasma samples from 73 patients with relapse of ovarian cancer shortly before start of second-line chemotherapy. Plasma YKL-40 was increased in ovarian cancer patients (median 94 µg/L, range 20–1970 µg/L) compared with age-matched controls (33 µg/L, range 20–130 µg/L) ( $p < 0.001$ ). High plasma YKL-40 is related to short survival in patients with recurrent ovarian cancer (Dehn et al., 2003). Plasma YKL-40 was also identified as an obesity-independent marker of type 2 diabetes related to fasting plasma glucose and plasma IL-6 levels (Nielsen et al., 2008).

Protein biomarkers can also be measured by LC-MS either in intact protein form (top-down) or by unique surrogate peptide generated after enzymatic digestion (bottom-up) (Liebler and Zimmerman, 2013; Percy et al., 2014). The enzyme digestion condition and selection of appropriate internal standards can have significant impact on the assay quality (Bronsema et al., 2013). The top-down approach usually uses high-resolution MS such as time of flight (TOF) MS while the bottom-up approach uses traditional multiple reaction mode (MRM). Oftentimes, immunoaffinity extraction using antibody of the target analyte is used to improve the assay selectivity and sensitivity (Carr and Anderson, 2008; Wang et al., 2012). Further selectivity/sensitivity enhancement can be achieved using dual immunoaffinity capturing procedure

as in the example of quantifying interleukin 21 (IL-21) (Palandra et al., 2013). An immunoaffinity LC-MS assay for quantification IL-21 in human and cynomolgus monkey serum was developed. The workflow includes offline enrichment of IL-21 using an anti-IL-21 capture antibody, followed by trypsin digestion, online enrichment of IL-21 derived tryptic peptides using antipeptide antibodies, and quantification using nanoflow LC-MS.

Apolipoproteins are high abundance serum proteins situated on the surface of lipoprotein particles that transport highly hydrophobic lipids. Current evidence suggests that ApoA-1 is a potential diagnostic biomarker for coronary heart disease risk (Rader and Hovingh, 2014). Furthermore, the risk of coronary heart disease is strongly associated with increased adiposity, which can be further increased by smoking behavior (Slagter et al., 2013). A stable isotope dilution LC-MS method for serum ApoA-1 was developed and validated. Full validation was performed by employing nine tryptic peptides generated from native ApoA-1 in order to maximize coverage of the endogenous ApoA-1 protein. Recombinant ApoA-1 internal standard was prepared by stable isotope labeling with amino acids in cell culture (SILAC) by using [ $^{13}\text{C}_6\ ^{15}\text{N}_2$ ]-lysine and [ $^{13}\text{C}_9\ ^{15}\text{N}_1$ ]-tyrosine (Wang et al., 2015).

Apolipoprotein C3 (ApoC3) is one of many plasma glycoproteins which have been extensively studied for potential utility as disease biomarkers. ApoC3 is a 79-amino acid protein synthesized by liver and intestine. ApoC-3 has a critical role in the metabolism of triglyceride (TG)-rich lipoproteins (TRLs) (Norata et al., 2015). Previously, an LC-MS assay using a solid-phase extraction (SPE) method for the plasma sample preparation was published. This “top-down” approach provided intact analysis of ApoC3 glycoisoforms and potential for data mining, and high-resolution MS afforded excellent specificity. The assay was also applied to analysis of plasma samples collected from normal, prediabetic, and diabetic subjects for preliminary evaluation of the biomarker potential of ApoC3 glycoisoforms for early diagnosis of diabetes. The results showed that there was a significant difference among the different groups (Jian et al., 2013).

### 1.8.2 Peptide Biomarkers

Peptides can be an important class of biomarkers. Traditionally, peptide biomarkers in biological samples have mostly been analyzed by immunoassay methods. Similar to protein biomarkers, cross-reactivity with structurally related peptides prevents selectivity. The combination of a separation technique such as micro/nano-HPLC with a detection method as MS is a very selective and sensitive approach and permits the

simultaneous analysis of a great number of peptides (Saz and Marina, 2008).

The 40- and 42-amino acid residue forms of  $\beta$ -amyloid ( $A\beta 1-40$  and  $A\beta 1-42$ ) in CSF have been proposed as potential biomarkers of AD (Whiley and Legido-Quigley, 2011). In 2006, an immunoaffinity purification and LC–MS assay was developed for analysis of amyloids in CSF (Oe et al., 2006). In another report, a mixed-mode SPE method and an ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) assay was developed for the simultaneous quantitation of  $A\beta 1-38$ ,  $A\beta 1-40$ , and  $A\beta 1-42$  from human CSF (Lame et al., 2011). Analysis of  $A\beta$  peptides in plasma has its own methodological challenges, including binding to plasma proteins and carryover of analytes from previous injections when using LC (Goda and Kobayashi, 2012).

There is also a substantial evidence that  $\beta$ -amyloid peptide is oxidized *in vivo*, which has led to the suggestion that oxidative stress might be an important mediator of AD. Trypsin digestion of both native and oxidized  $A\beta 1-16$  and  $A\beta 1-40$  resulted in the formation of tryptic peptides corresponding to native and oxidized  $A\beta 6-16$ , which could be separated by LC. Sites of oxidation were then unequivocally characterized as histidine-13 and histidine-14 by LC–MS analysis of the tryptic peptides (Inoue et al., 2006).

### 1.8.3 RNA Biomarkers

Micro RNAs (miRNAs) are small noncoding RNAs found in eukaryotic organisms that regulate gene expression. Dismissed as “junk” until about a decade ago, it is now widely accepted that they play an important functional role in a wide array of cellular processes. miRNAs play important regulatory roles in many cellular processes, including differentiation, neoplastic transformation, and cell replication and regeneration. Many studies have demonstrated that dysregulation of these miRNAs is associated with various diseases suggesting there is potential for use of miRNAs in diagnosis and treatment. Arguably, secreted miRNAs have many requisite features of good biomarkers. They are stable in various bodily fluids, the sequences of most miRNAs are conserved among different species, the expression of some miRNAs is specific to tissues or biological stages, and the level of miRNAs can be easily assessed by various methods, including methods such as PCR, which allows for signal amplification. Much of the study of miRNA and disease has focused on cancer and neurological disorders (Ju, 2010). A number of bioanalytical challenges exist for the analysis of miRNAs in biological fluids as very nicely summarized by Wang and Ji (2016) and by Basiri and Bartlett (2014). While the PCR and hybridization ELISA give the best sensitivity, LC–MS shows promise to be the next

generation analyzer for this type of molecules due to its sensitivity for small oligonucleotides (<25-mer), broader dynamic range (up to 3 orders of magnitude), no need for specific reagent, and the capability to quantify intact double-stranded oligonucleotides (siRNA) and their metabolites.

### 1.8.4 Nucleotide Biomarkers

Plasma concentrations of nucleotides such as AMP, ADP, and ATP provide information on their relative physiological importance in regulatory mechanisms and therefore could be useful biomarkers. Analytical approaches of determining AMP, ADP, and ATP in biological samples have been proven challenging due to their high polar nature. Zhang et al. discussed a novel, fast, highly sensitive, selective, and validated ion-pairing hydrophilic interaction chromatography (HILIC)–MS method utilizing diethylamine (DEA) and hexafluoro-2-isopropanol (HFIP) in the mobile phase and an aminopropyl chromatographic column (Zhang et al., 2014).

### 1.8.5 Small Molecule Biomarkers

One of the great promises of the metabolomics approach is the fact that groups of metabolite biomarkers are expected to be less species-dependent than gene or protein markers, facilitating the direct comparison of animal models with human studies, which in turn improves the potential of the technique to rapidly convert laboratory-based research into clinical practice (Barr et al., 2010). Typically, LC–MS-based technologies are used for small molecule biomarker analysis. Challenges of analyzing small molecule biomarkers include separation of isobaric position isomers which have identical molecule weight to the analyte of interest, poor retention due to extremely polar nature, poor sensitivity due to lack of favorable ionization function groups, and poor stability. Derivatization strategy has been frequently used to enhance sensitivity and selectivity (Meyer et al., 2011). Novel chromatographic stationary phase such as HILIC can be used to enhance the retention and thus the sensitivity of polar biomarkers (Weng, 2001; Jian et al., 2011; Li et al., 2012). Care should also be exercised to prevent introducing artifacts during the sample storage and processing (Chao et al., 2008).

Cytochromes P450 3A4 (CYP3A4) and CYP3A5 are important human drug metabolizing enzymes with high interindividual variability in hepatic and intestinal activities. DDI with CYP3A4-inhibiting drug such as itraconazole or inducing drug such as rifampin can dramatically change the CYP3A4 activity in man. Therefore, regulatory agencies such as FDA and EMA have issued guidelines on assessing DDI mediated by P450 enzymes

including CYP3A4, 2D6, 2C9, 2C19, and so forth, and various transporters. The most widely used and accepted method to assess CYP3A activity is to examine midazolam PK. Urinary 6 $\beta$ -hydroxycortisol to plasma cortisol metabolic ratio has also been used historically as a non-invasive measure of CYP3A activity (Lutz et al., 2010), which is a more rapid biomarker due to short half-life with little delay time behind the changes of CYP3A4 activity *in vivo*. However, diurnal effect leads to more variable data. Plasma 4 $\beta$ -hydroxycholesterol is an endogenous metabolite of CYP3A4-mediated cholesterol metabolism and has been extensively investigated. It is the first choice if a stable biomarker is needed. The long half-life of 4 $\beta$ -hydroxycholesterol results in small variations in its concentrations but excludes this marker in short-term studies. Using both biomarkers in clinical studies would be recommended if the outcome is unknown (Märde Arrhen et al., 2013; Dutreix et al., 2014).

24S-hydroxycholesterol (24S-HC) can be formed from cholesterol via cytochrome P450 family 46A1 (CYP46A1, cholesterol 24-hydroxylase) in brain. 24S-HC is capable of passing across the blood–brain barrier and enters the systemic circulation. Therefore, the plasma concentration of 24S-HC can be used as a marker for cholesterol homeostasis in the human brain (Lutjohann et al., 1996). Sugimoto et al. reported a highly sensitive and specific LC–MS method with an atmospheric pressure chemical ionization interface to determine 24S-hydroxycholesterol in plasma (Sugimoto et al., 2015). Phosphate-buffered saline including 1% Tween 80 was used as the surrogate matrix for preparation of calibration curves and quality control samples. The saponification process to convert esterified 24S-hydroxycholesterol to free sterols was optimized, followed by liquid–liquid extraction using hexane. Chromatographic separation of 24S-hydroxycholesterol from other isobaric endogenous oxysterols was successfully achieved with gradient elution on a C18 column. This assay was capable of determining 24S-hydroxycholesterol in human plasma (200  $\mu$ L) ranging from 1 to 100 ng/mL with acceptable intra- and interday precision and accuracy.

## 1.9 Conclusion and Future Perspective

There is no doubt that mass spectrometry-based technologies will continue playing major roles for biomarker research including quantitation, especially for small molecule biomarkers and peptide biomarkers which arguably provide more direct links to a biological process *in vivo* since many of these small molecules or peptides are the direct substrates of these biological processes. There are also many mature technologies

available and wealthy application information from literature. Seldom did we fail to develop an assay to quantify this type of biomarkers even though some of them can be quite challenging. We will continue see the use of fit-for-purpose approach in the assay establishment so that the right resources and costs are utilized at different stages of drug discovery and development programs. Continual dialog between industry and regulatory authorities will lead to better and more practical solutions on biomarker quantitation.

Challenges are still ahead of us. Both protein and miRNA biomarkers present significant challenges for LC–MS bioanalysis. Proteins can be measured by LBAs but they suffer potential cross-reactivity with similar proteins which may exist in much high quantity in the samples. Current bottom-up approach (use of surrogate peptide after enzymatic digestion to reflect the intact protein biomarker), while more sensitive than the top-down approach, requires extensive method development and thorough understanding of structure modifications of protein in the body. The top-down approach which utilizes the high-resolution MS detection is less subject to quantitation bias due to protein modifications but is significantly less sensitive and is currently only limited to abundant protein biomarkers. miRNA can be measured by qPCR but the procedure is tedious and assay accuracy is less than desirable to support biomarker utilization with small to moderate changes. Attempt of using LC–MS for miRNA biomarkers is made but all these LC–MS assays suffered from poor sensitivity due to unfavorable ionization of RNA type of molecules, compounded by use of ion-pair reagents or high level of buffers in the mobile phases, typically for RNA/DNA molecules in order to maintain good peak shape.

We will continue to see the improvement of sensitivity by using sample preparation technologies such as immunoaffinity extraction which not only allows cleaner extraction but also provides analyte enrichment. Currently, it is quite costly to use such an approach. Hopefully with more commercialization of antibodies and more automation, the cost will come down significantly. We will also see the use of more applications and refinements of using nano- or micro-LC for the sensitivity enhancement. The lack of system robustness and ability of swift switch of assay parameters are the current limitations, especially for the nano-LC system, which prevents the full utilization of such systems in support of discovery programs where the same instrument needs to support multiple programs with much diversified chemical structures. We will also see some of the enhancement of additional separation capabilities such as ion-mobility device that can assist in separating isomers in the ionization sources. Nevertheless, the Achilles heel of LC–MS-based technologies is the inadequate

sensitivity to measure low abundant protein or RNA biomarkers. In order for LC–MS to be more universally applicable to quantifying protein and RNA biomarkers,

the absolute sensitivity of mass spectrometer, especially high-resolution mass spectrometer, must be significantly improved.

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## 2

### Translational Application of Biomarkers

*Ray Bakhtiar*

*Teva Branded Pharmaceutical Products R&D, Inc., West Chester, PA, USA*

#### 2.1 Introduction

Drug discovery and development is a lengthy, costly, and high-risk endeavor owing to its scientific and regulatory complexities. The emergence of new biomarkers, personalized medicine, and technological advancements has led to innovative breakthrough therapies for larger segment of patients at a global scale (Mignani et al., 2016). Government laboratories, academic institutions, and pharmaceutical corporations have established partnerships to address complex unmet needs to refine risk versus benefit evaluations from preclinical to clinical proof of concept (Fishburn, 2013). These partnerships have been in the form of fee-for-service agreements, unrestricted grants, corporate venture capital funds, corporate discovery centers, or licensing agreements. With the advent of the Bayh–Dole Act or Patent and Trademark Law Amendments Act of 1980, government-funded research has been able to move into the commercialization space of biomedical sciences and promote public health objectives. Consequently, pioneering accomplishments have started to be realized in recent years. For example, with respect to the number of biologics license applications (BLAs) and new drug applications (NDAs), 2015 had the second highest number of BLAs and NDAs since 1998 at 13 and 32 approvals, respectively (Morrison, 2016).

In order to further improve the deficiencies in study designs, address the heterogeneity of drug response in humans, and address remaining challenges facing the traditional bench-to-bedside continuum, areas such as translational medicine (TM) have taken a central role in today's discovery and development of first-in-class products (Fuster, 2014; Howells et al., 2014). In this chapter, we present a brief overview of TM and its relation with respect to discovery and application of preclinical and clinical biomarkers.

#### 2.2 Translational Medicine

TM is referred to interdisciplinary efforts between research and development groups to bridge the gap between basic and clinical sciences by exploiting innovative tools and technologies. The ultimate objective of TM is to efficiently identify and validate novel biological disease targets, discover viable molecules to engage those targets, seek regulatory approval, and commercialize the products for use (Mullane et al., 2014).

Although the term translational or personalized medicine has emerged as the new “buzzword” in recent years, TM itself is actually not new. In the 1950s, Arvid Carlsson, a Swedish scientist, performed a series of elegant experiments in rabbits treated with reserpine before and after administration of levodopa (L-DOPA), which ultimately led to the first clinical treatment for Parkinson's disease (PD), a therapy that still continues to be widely used (Andersen, 2009). Carlsson showed that dopamine was a neurotransmitter in mammalian brain and administration of L-DOPA to dopamine depleted rabbits could lead to regaining lost motor functions (Carlsson et al., 1957). In 2000, the Nobel Prize in Physiology or Medicine was awarded jointly to Arvid Carlsson, Paul Greengard, and Eric R. Kandel for their discoveries concerning signal transduction in the nervous system.

Of course, one of the key determinants of success is how well a validated in vitro or animal model can closely mimic the complex biology of human response in a clinical setting (Sultana et al., 2007). One practical approach has been to identify and validate a biomarker, in conjunction with genetic information, with the goal to ultimately identify the right drug, at the right dose, at the right time, and for the right patient (Aronson et al., 2008; Wehling, 2009; Terzic and Waldman, 2010; Fiore and D'Avolio, 2011).

## 2.3 Biomarkers

In 2001, with the aim to harmonize basic terminologies for biomarkers, the US National Institute of Health (NIH) Working Group on Definitions recommended the following (Biomarkers Definitions Working Group, 2001):

- Biological marker (biomarker): A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.
- Clinical end point: A characteristic or variable that measures how a patient feels, functions, or survives.
- Surrogate end point: A biomarker that is intended to substitute for a clinical end point. A surrogate end point is expected to predict clinical benefit (or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence.

The biomarker science is a multifaceted discipline with broad utilities in diagnosis, toxicity, prognosis, efficacy, and stratification applications (Bakhtiar, 2008; Zhao et al., 2015). Even within these broad categories, there are distinct molecular types of biomarkers, such as:

- Genomic biomarkers that involve deoxyribonucleic acid (DNA) patterns as well as mining single nucleotide polymorphisms (SNPs)
- Transcriptomic biomarkers that are snapshots of ribonucleic acid (RNA) expression levels
- Proteomic biomarkers that involve analysis of the protein expression levels
- Metabolomic biomarkers that are identification and determination of metabolites that are specific to cellular processes

According to [www.clinicaltrial.gov](http://www.clinicaltrial.gov), accessed on March 7, 2016, there have been 17,838 clinical trials that utilize biomarkers. However, biomarkers can be heterogeneous in nature and expressed at relatively low levels in systemic circulation or tissues, posing technical challenges. Their biology may not be well understood, their response may not parallel drug and/or its active metabolite, sampling may require invasive procedures, and their utility may require careful planning in biomarker assay and therapeutic agent co-development (Colburn, 2003; Wehling, 2006; Spinella, 2009).

Criteria of an ideal biomarker include (Frangogiannis, 2012; Cohen et al., 2015):

- Translates from the preclinical to clinical space.
- Present at relatively high concentration in bodily fluid to sample using noninvasive or minimally invasive procedures.

- There is a clear dose–response relationship.
- Yields a consistent response across different studies.
- Robust link between marker and the disease state.
- Yields additional information to existing knowledge.
- Assists the healthcare professional in critical clinical decisions.
- Availability of a reliable and sensitive assay, preferably noninvasive, for determination of the marker.
- Changes with statistically significant margins versus the control cohort.
- Data that are reproducible across laboratories by different investigators.
- Sampling and analysis is cost effective and rapid.

With the above in mind, the ideal biomarker can then be integrated into a number of areas such as system biology, modeling, bioinformatics, trial simulation, clinical trial designs, and personalized medicine treatment (Krishna et al., 2007).

In recent years, a new paradigm has evolved, known as model-based drug development (MBDD), which uses statistical and mathematical tools to combine the knowledge obtained from early discovery to post-marketing (Suryawanshi et al., 2010). In MBDD, biomarkers play an integral role in quantitative integration of information obtained from preclinical models through late-stage clinical trials and ultimately post-marketing commitments. The six components of MBDD are trial performance metrics, quantitative decision criteria, data analysis model, competitor information and meta-analysis, clinical trial design model, and pharmacokinetic/pharmacodynamic (PK/PD)/disease model (Lalonde et al., 2007). The pharmacokinetic/pharmacodynamic (PK/PD)/disease model is heavily dependent on a validated biological marker that could construct a temporal relationship between drug dose, exposure, and response. Table 2.1 summarizes the five phases of biomarker development, which conforms to the traditional framework of drug discovery and development (Pepe et al., 2001).

## 2.4 Biomarker Categories

There are a myriad of conference workshop reports and published manuscripts that attempt to categorize biomarkers and their applications from discovery to post-marketing clinical studies. An example of biomarker categories is presented in Table 2.2. Each of these categories could apply to a preclinical model as well as human, if translatable. In this regard, a challenge is in the case of first-in-class or novel chemical entities, leading to breakthrough therapies, whose mechanisms of action (MoAs) are not fully understood. Consequently, translation from preclinical species to human physiology may benefit

**Table 2.1** Five phases of biomarker development.<sup>a</sup>

Stage	Study design	Phase	Example of specific objectives
Discovery	Preclinical/exploratory	I	Promising directions identified
Translational across animal test species and into the clinic	Clinical assay and method validation	II	Detect established biomarker
	Retrospective longitudinal	III	Biomarker detects outcome early prior to becoming clinically apparent
Clinical validation	Prospective screening	IV	Application to a wider cohorts of subjects to identify false positives
		V	Impact of screening on the population is quantified

<sup>a</sup> Pepe et al. (2001).

**Table 2.2** Simplified biomarker categories in drug discovery and development.

Biomarker category	Definition	Examples of application(s)	Biomarker example(s)
Toxicity	Exclude certain patients from treatment; monitor and avoid severe adverse events	Cardiotoxicity due to chemotherapy	Screening of left ventricular ejection fraction (LVEF) using radionuclide ventriculogram (MUGA) or 3D echocardiography; troponin T and I cardiac proteins following myocardial injury
Efficacy	Establish dose-response curve; to indicate a change in the disease state during the treatment phase	Measuring drug-related receptor occupancy (RO) with positron emission tomography (PET)	Brain kinetics of methylphenidate enantiomers after oral administration
Prognostic	Identify the likely course of the disease in untreated cohorts	Stage II and III colon cancer	Caudal type homeobox transcription factor 2 (CDX2)
Predictive/early diagnostic	Identify the most at-risk subjects to detect the disease while measurable and yet not clinically apparent	<i>In vivo</i> imaging of the cortical regions of the brain as an early detection of Alzheimer's disease	Deposition of β-amyloid
Stratification	Select best treatment for each subject	Adjuvant and metastatic breast cancer; metastatic cancer of the stomach or gastroesophageal junction	Human epidermal growth receptor 2 (HER2) testing prior to treatment with trastuzumab

from predictive biosimulation to better design a clinical protocol (Kansal and Trimmer, 2005). In some other instances, a simulation and modeling approach may not fully address the gaps posed by lack of a clinically validated animal model. These include complex neurological disorders such as ALS, for which predicting a patient's prognosis has not improved over the last seven decades. Other areas of difficulty include translation of knowledge regarding a disease root cause, onset, and progression (Barker-Haliski et al., 2014).

A good toxicity biomarker establishes consistent association between the marker and early indications of toxicity (Sistare and DeGeorge, 2011). In addition, an ideal toxicity biomarker provides a high level of

concordance between clinical adverse effects (AEs) and observations in preclinical safety assessment models. For example, blood levels of aspartate transaminase (AST) and alanine transaminase (ALT) enzymes have been often used as markers of liver injury. However, ALT and AST are not predictive of the overall liver function. A number of other markers are currently being evaluated including glutamate dehydrogenase (GLDH), malate dehydrogenase (MDH), glutathione-S-transferase (GST), and arginase (ARG-1) (Campion et al., 2013; Schomaker et al., 2013). Another example would be a biomarker of acute renal injury, which has been on the rise and of a global concern (Lameire et al., 2013). Although serum creatinine (SCr) and blood urea nitrogen (BUN) have been

among the traditional renal toxicity markers, they are not entirely reflective of the glomerular filtration rate (GFR) and lack the ability to differentiate between different stages of damage. To this end, promising data on kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), hepatocyte growth factor (HGF), and total protein data have led to optimism in using a combination approach to decipher a more comprehensive understanding of acute kidney injury (AKI) (Campion et al., 2013; Coca and Parikh, 2008).

An efficacy biomarker establishes a strong link between the target and the disease, is used to establish a dose–efficacy response curve, and yields a high degree of confidence of its reliability for phase III clinical trials (Bradley, 2012). Despite notable advances in our understanding of a host of diseases and their molecular basis, there remains to be a paucity of robust early efficacy markers for clinical uses (Gobburu and Lesko, 2009). An established approach has been to link target engagement to a PD marker using translational imaging techniques (Hargreaves and Rabiner, 2014). In psychiatric diseases, positron emission tomography (PET) technique that utilizes radionuclide-containing imaging agents (e.g., <sup>11</sup>C, <sup>18</sup>F, etc.) has proven to be a versatile tool in preclinical and clinical research. Clinical application of imaging techniques has been on the rise due to their noninvasive nature and thus compatibility with human clinical trials. The use of an appropriate radioligand that exhibits high sensitivity and contrast between the treatment and diagnostic cohort, yet remains unaffected by imaging software, hardware, or nonclinically related intra-subject variability, is ideal (Linden, 2012). Dose titration coupled with imaging techniques can establish a correlation between receptor occupancy (RO) and systemic exposure in order to test doses administered during clinical studies. For example, a clinical study using PET imaging involving an investigational neurokinin I (substance P) receptor antagonist, L-759274, for the treatment of anxiety disorder reported lack of efficacy in spite of 90% RO at steady-state plasma concentration of L-759274 (Michelson et al., 2013). Lastly, another commonly used efficacy biomarker is hemoglobin A1c (HbA1c), which is used in trials of investigational drugs as an indication of glycemic control in diabetic patients.

A prognostic biomarker assists investigators to identify the best course of treatment to indicate long-term outcome for patients and yields an estimate of the severity and the likely outcome of the disease. For instance, inflammatory prognostic biomarkers have shown clinical value in various cardiovascular indications. C-reactive protein (CRP) has now been established as a prognostic marker for vascular disease, inducing the upregulation of cellular adhesion molecules (Ridker, 2001). As a result, the outcomes of clinical

studies have shown that statin treatment can lower the CRP to <2 mg/L, leading to event-free survival (e.g., decrease in risk of reoccurrence of myocardial infarction (MI) in subjects with a history of acute coronary syndrome (Ridker et al., 2005).

Predictive or early diagnostic markers are used for early detection of disease signals that are indicative of subjects most at risk while measurable and yet not clinically apparent. SNPs and haplotypes have been used to decipher the genetic basis of a number of human diseases for advance diagnosis and detection. Microarray and differential gene expression have been used to determine up- and downregulation of genes (Bai and Abernethy, 2013). Extensive research has been devoted to identification of predictors of Alzheimer's disease (AD) and other types of dementia. Some of the more promising markers have been β-amyloid (1–42), total tau, and phospho-tau 181 in cerebrospinal fluid (CSF) using immunoassay techniques (Humpel, 2011).

A stratification marker is important for selecting the best treatment for each patient by prospectively identifying them. In such a case, patients are stratified into biomarker-positive and biomarker-negative strata. This approach is also referred to as “enrichment” and often increases the possibility of treatment benefits to the biomarker-positive subpopulations and unnecessary side effects to biomarker-negative ones. The biomarker can be measured either at the clinical site using an approved companion or in vitro diagnostic (IVD) kit or shipped to a laboratory for more complex measurements. An IVD should ideally be developed contemporaneously with its respective therapy so the patient data generated can assist in the validation process. For example, trastuzumab, a humanized monoclonal antibody, is a human epidermal growth factor receptor 2 (HER2) antagonist, where the HER2 is overexpressed. HER2 is a modulator of cell proliferation and growth and its overexpression occurs in about 20% of breast cancer patients. Targeting HER2 with trastuzumab has improved the survival rate of patients with metastatic and early HER2-positive breast cancer. Therefore, patients that are likely to respond to trastuzumab therapy are selected based on approved methods for HER2 gene amplification, such as immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH), for protein overexpression and gene amplification measurements, respectively (Kelloff and Sigman, 2012). Another similar case is gefitinib, a small molecule tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR). Originally, gefitinib was unsuccessful in treating unselected patients. However, it showed promising results and was approved for use as the first-line therapy in patients with metastatic non-small cell lung cancer (NSCLC) whose tumors have EGFR exon 19 deletions or exon 21

substitution mutations as detected by an approved test (Beckman et al., 2011).

In the following sections, specific examples of biomarkers and their role in TM in several active areas of clinical research will be presented.

## 2.5 Neurobiological Disorders

The human brain is a sophisticated and complex information processing machine. Consequently, discovery and validation of biomarkers in neuroscience remain challenging with a number of areas unanswered. Areas of interest include predictive neuroscience, understanding the neuronal network and signaling, memory storage, and data processing. Several neurological disorder risk factors increase with advanced age. Two such prominent diseases, particularly in the aging population, are AD and PD. According to the US Census Bureau, by 2050, the population aged 65 and over is anticipated to be about 83.7 million, roughly double its estimated population over 65 of 43.1 million in 2012. Furthermore, according to the United Nations, globally, the number of older individuals that are aged 60 years or higher is expected to more than double, from 841 million people in 2013 to more than 2 billion by 2050. By 2047, for the first time, older individuals are anticipated to exceed the number of children.

Thus far, therapeutic approaches for neurobiological disorders have been mostly limited to the management of their symptomatic aspects. Hence, early detection of the central nervous system (CNS) diseases could offer possibilities of better treatment and clinical outcomes for AD, PD, depression, pain, and multiple sclerosis. For preclinical drug discovery studies, particularly in rodents, brain tissues are harvested and analyzed. However, for clinical studies, CSF sampling has been used to indirectly measure drug or biomarker concentrations. For PD biomarkers, Lewy body inclusions mostly consisting of the protein  $\alpha$ -synuclein monomeric and oligomeric forms have been implicated in this disease. A host of CSF proteins such as apoE, apoAII, vitamin D binding protein (VDBP), IL-8, and brain-derived neurotrophic factor (BDNF) have shown to be promising in diagnosis of PD patients. Challenges that remain, include correlation between CSF and brain protein biomarker concentrations, early or presymptomatic diagnosis, and disease progression monitoring (Flood et al., 2011; Chen-Plotkin, 2014). In addition, brain imaging techniques have shown utility in pre- and postsynaptic dopamine receptor evaluation in the striata of PD patients (Wong et al., 2007).

Up to 120 gene loci related to AD have so far been identified, and 4 of them appear to have links to this

disease, including APP, APOE, PSEN1, and PSEN2 (Flood et al., 2011). Other well-studied AD biochemical biomarkers include increases in levels of total tau and CSF phosphorylated tau isoforms (e.g., six varying in number of amino acids from 352 to 441) that increase with neurodegeneration advancement. Decreased CSF  $\beta$ -amyloid 42 (A $\beta$ 42) could also be related to cognitive deterioration due to its fibrillary disposition in diseased brains. The above associations have been in line with the results obtained from amyloid PET imaging and 2-[<sup>18</sup>F] fluoro-2-deoxy-D-glucose (FDG) hypometabolism in brain's temporal and parietal lobes. Currently, there are three approved tracers for AD diagnosis used for amyloid neuritic plaque density imaging (George et al., 2015). Therefore, a simplified proposed cascade of events includes accumulation of A $\beta$ 42, tauopathy, neurodegeneration, and ultimately clinical symptoms (Jack and Holtzman, 2013). Several interdependent approaches remain central for the diagnosis and progression assessment of AD, such as determination of CSF biomarkers, brain metabolism by imaging techniques, and behavioral observations.

Search for robust translational PD biomarkers in schizophrenia and multiple sclerosis, in particular for early diagnosis, continues to be an active area of research. For example, in schizophrenia patients, while no clinical brain imaging technique exists, this approach is utilized to diagnose multiple sclerosis by looking for the presence of lesions or telltale scars on the brain or spinal cord (Wong et al., 2007). PET and a high-resolution modality known as single-photon emission computed tomography (SPECT), albeit less sensitive than PET, have aided in understanding the pathophysiology of schizophrenia. Other techniques include translation of signals obtained from electroencephalography (EEG) from preclinical to clinical space with regard to patients' deficit in EEG frequency band power in neuronal pathways (English et al., 2014). Translatable CSF and plasma biomarkers for schizophrenia have proved to be elusive, yet there are promising signs of progress. For example, the S100b calcium-binding protein, which plays a role in cell proliferation and astrocyte activation, has shown elevated levels in schizophrenia subjects correlating with cognitive impairment observed in chronic cases (English et al., 2014). Body fluid biomarkers have also been studied, in addition to magnetic resonance imaging (MRI), to diagnose multiple sclerosis. Part of the multiple sclerosis research efforts has focused on the discovery and validation of biomarkers for conversion and diagnosis of clinically isolated syndrome (CIS) to multiple sclerosis. CIS refers to the early clinical stages of CNS demyelination that can precede the clinically definite multiple sclerosis (CDMS) period. Elevated levels of CSF-specific IgG oligoclonal bands (OCBs), IgM-OCBs, and chitinase-3-like

protein 1 (CHI3L1) have shown promise to support early diagnosis and identification of CIS converters to multiple sclerosis (Teunissen et al., 2015).

## 2.6 Cardiovascular Disorders

Development and clinical applications of biomarkers in cardiovascular diseases (CVDs) have made substantial progress in the last several decades. Biomarkers such as blood pressure, cholesterol, triglyceride, fibrinogen, cardiac troponins I and T, and CRP have facilitated diagnosis, early decision making, and clinical treatment. In parallel, research has been directed to classify the effects of drugs on epigenetic changes (epigenome); in RNA modifications (epitranscriptome), protein expression (transcriptome), and other molecules (metabolome); in polypeptides (proteome); and in all their interactions (van Holten et al., 2013; Friso et al., 2015).

Coronary CVD mortality is often related to the atherosclerotic plaque formation and rupture, which leads to vascular occlusion and thrombosis. Early detection of molecular signatures, causative mechanism of plaque rupture, and pharmacogenomic factors will aid in the prevention and treatment of such a disease. CRP, lipoprotein-associated phospholipase A2 (Lp-PLA2), and myeloperoxidase (MPO) are among the approved cardiovascular biomarkers by the US Food and Drug Administration (USFDA). In addition, currently two other circulating biomarkers are being investigated for possible early detection of cardiotoxicity (Dadu et al., 2012; Sawaya et al., 2012).

CRP, an inflammatory marker, has been implicated in atherosclerosis by facilitation of cell surface adhesions, reduction of nitric oxide activity, and increasing the uptake of oxidized low-density lipoprotein (LDL) cholesterol by macrophages and transformation of the latter into foam cells. This process then yields to generation of fatty streaks on the blood vessel walls, inflammation, and ultimately atherosclerotic lesions. In the clinical arena, a high-sensitivity CRP (hs-CRP) test is used in the evaluation of CVD, which could be combined with a lipid profile or with other cardiac risk markers, such as Lp-PLA2 test, to provide added information about heart disease risk. The hs-CRP levels of <1, 1–3, and >3 mg/L are clinically suggestive of lower, moderate, and higher relative risk of CVD (Dadu et al., 2012). Currently, there are no drugs that target CRP; however, a number of clinical trials have been designed to investigate a candidate drug's anti-inflammatory effect, leading to a reduction in CRP levels.

Lp-PLA2 is a 50kDa enzyme that is produced by inflammatory cells. It has been proposed that Lp-PLA2 may yield lysophosphatidylcholine and oxidized fatty

acid products, subsequently downregulating nitric oxide release and upregulating CD40 ligand expression in T cells, thus leading to a higher risk in coronary heart disease (Dadu et al., 2012). In general, levels of Lp-PLA2 of <200 and >235 ng/mL have been associated with low and high risks of CVD, respectively. Lp-PLA2 levels have been shown to decrease after treatment with statins, niacin, fenofibrate, and omega-3 fatty acids (Dadu et al., 2012).

MPO is another protein that is present at higher levels under inflammatory conditions. MPO is generated by macrophages and neutrophils, leading to oxidation of lipids within the LDL particles yielding foam cells. MPO levels of >350 µg/L pose concern for a higher incident of MI. Lifestyle changes and statin therapy have shown to reduce MPO levels (Dadu et al., 2012). However, additional well-controlled clinical studies are needed to further establish the validity of MPO as a biomarker for CVD management.

Cardiotoxicity has been established as one of the AEs of cancer chemotherapy (Stevens and Lenihan, 2015). For example, combination of trastuzumab and anthracycline therapy has shown to cause left ventricular (LV) irregularities in up to ~33% of cancer patients (Sawaya et al., 2012). The current clinical method of evaluation for cardiotoxicity detection is assessment of left ventricular ejection fraction (LVEF) using echocardiogram (contrast, tissue Doppler imaging, and/or 3D) and radionuclide ventriculogram (MUGA). According to the American Heart Association and American College of Cardiology guidelines, use of cardioprotective drugs in asymptomatic patients with abnormal LVEF (e.g., LVEF values of <50%) can be considered. However, there are several disadvantages with the above cardio-imaging approaches, including low sensitivity, cost, variabilities between observers, and low sensitivity in detection of early clinical signs. To that end, there has been a degree of enthusiasm to discover and biologically validate circulating biomarkers for earlier assessment of cardiotoxicity and use in conjunction with routine LVEF measurements.

Cardiac troponins T (cTnT) and I (cTnI) and brain natriuretic peptide (BNP) could be such biomarkers for early determination of cardiac injury. Cardiac TnT and TnI are cytosolic proteins that are present in heart muscles and are released into the bloodstream upon cardiac injury at relatively low (pg/mL) levels. Hence, sensitive and robust methods are needed to determine increases in the levels of cTnI and cTnT against a relatively substantial baseline. For instance, elevated levels of >30 pg/mL of cTnI have shown to be predictive of cardiotoxicity (Yu and Ky, 2016). BNP is a 32-amino acid peptide that is formed in the ventricular myocardium and is released upon ventricular filling pressure. Initially, the BNP

precursor is divided into the active moiety, BNP, and an N-terminal fragment known as NT-proBNP. Systemic measurements of BNP or NT-proBNP have been investigated in a number of clinical trials for early detection of LV irregularities and risk segregation among patients (Dadu et al., 2012). For instance, a BNP level of >100 pg/mL in cancer patients was proposed as a predictive tool for cardiac failure (Yu and Ky, 2016). Some of the current gaps include lack of universality, since a larger proportion of clinical studies have been performed in female cancer patients (e.g., breast cancer) so additional data on men are required; no clear consensus on cutoff values; and finally no general guidance on timing for biomarker sample collection (Stevens and Lenihan, 2015).

## 2.7 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is characterized as a condition involving airflow limitations that can be irreversible and related to lung inflammatory response. Genetic and environmental factors have been implicated in affecting the course of COPD. The early detection of COPD has remained to be a challenge, and extensive research effort is being directed in discovery and biological validation of new biomarkers. Currently, the gold standard for diagnosis and staging of COPD is forced expiratory volume in 1 s (FEV<sub>1</sub>) using spirometry, where a recommended value of FEV<sub>1</sub>/FVC<0.70 (forced vital capacity) is used for diagnosis (Ambade et al., 2015). Overall, the current COPD biomarkers suffer from several disadvantages: (i) spirometry cannot easily be performed by all patients since some cannot exhale for up to or more than 6s; (ii) FEV<sub>1</sub> while suitable for prognosis purposes, they can be imprecise in addressing the underlying pathological activity; (iii) they do not distinguish phenotypes of the disease; (iv) they lack specificity to COPD; and (v) they remain unmodified by some therapies that improve survival, such as long-term oxygen therapy.

Clearly there is a need to identify robust and reliable COPD biomarkers that could also distinguish COPD subgroups and their respective treatments. In 2009, Sin and Vestbo outlined a series of proposed criteria for developing novel biomarkers or surrogates for COPD (Sin and Vestbo, 2009):

- Is there a strong biological plausibility in terms of its role in the pathogenesis of COPD?
- Is there a strong, consistent, and independent association between the biomarker and COPD?
- Is there a strong independent association between the biomarker and hard clinical outcomes such as mortality and hospitalization?

- Is there evidence from randomized controlled trials that the biomarker is modifiable by interventions?
- Is there evidence from randomized controlled trials that changes in the biomarker status result in changes in an important (and accepted) clinical outcome (e.g., mortality, exacerbation, rate of decline in FEV<sub>1</sub> and health status)?

In 2010, to address the above challenges in the development and validation of new COPD biomarkers, the COPD Foundation established the COPD Biomarker Qualification Consortium (CBQC). The CBQC is a partnership between the COPD Foundation, the pharmaceutical industry, and the USFDA to harmonize industry and publicly funded data and to facilitate such endeavors (Casaburi et al., 2013). To that end, biomarkers such as interleukin 6 (IL-6), interleukin 8 (IL-8), surfactant protein D (SP-D), and fibrinogen have shown promising results.

Tocilizumab is a humanized IL-6 receptor-inhibiting monoclonal antibody that has been approved for moderate to severe rheumatoid arthritis in adults because patients showed elevated IL-6 levels in serum and synovial fluid (Alten, 2011). However, elevated IL-6 and IL-8 levels have also been detected in patients with COPD. IL-6, a small glycoprotein of ~21 kDa, plays a role in the regulation of inflammatory cells and is formed in the epithelium airway, macrophages, and other cells in reaction to cell stress. High levels of IL-6 have been linked to a lower FEV<sub>1</sub> in patients suffering from bronchitis and asthma (Chu et al., 2015). IL-8, ~8.5 kDa inflammatory cytokine, is formed by endothelial cells, mononuclear phagocytes, fibroblasts, and a number of other epithelial cells. IL-8 has potent chemoattractive and activating functions for neutrophils. IL-8 can prime the resting neutrophil, which is then dispatched to the site of inflammation and endothelium adhesion, and trigger histamine and leukotriene release from basophils (Tajima et al., 2009; Wright et al., 2010). Consequently, IL-8 can induce contraction of airway smooth muscle cells. IL-6 and IL-8 have shown to be sensitive biomarkers for detection of lung disease (e.g., in the sputum) (Koutsokera et al., 2013); however, their drawback has been the lack of specificity for lung disease and its acute injury (Leung and Sin, 2013; Allen and Kurdowska, 2014).

Alternatively, SP-D, a glycoprotein responsible for the regulation of innate immunity in the lung, has emerged as a potential viable biomarker for COPD. SP-D is located in the endoplasmic reticulum of type II pneumocytes and the secretory glands of Clara or club cells in the lungs (Kristan, 2013; Ambade et al., 2015). SP-D translocates to the systemic circulation in cases of increased lung permeability such as in COPD and therefore can be measured in the serum and sputum (Moreno et al., 2014).

There has been compelling evidence of elevated levels of SP-D in subjects with COPD prior to administration of oral corticosteroids as well as in smokers (Kristan, 2013; Ambade et al., 2015).

Blood fibrinogen has been associated with the presence of COPD (Mannino et al., 2015). A number of cross-sectional studies have shown that blood fibrinogen levels are higher in individuals with COPD compared with controls (Duvoix et al., 2013). Several methods have been used to determine fibrinogen levels including the Clauss method that measures thrombin-clottable protein in plasma samples and a direct method that measures immune-reactive fibrinogen protein. In that regard, plasma fibrinogen  $\geq 350$  mg/dL was associated with an increased risk of subsequent hospitalized COPD exacerbations (Mannino et al., 2015). Ultimately, the complexities of COPD and its progression do not allow the use of a single biomarker; hence, multiple markers will be needed to perform early diagnosis, monitor disease progression, and stratify patients for an effective treatment (Rosenberg and Kalhan, 2012).

## 2.8 Oncology

Arguably, an area that has considerably benefited by the advancement of biological markers is cancer research (Modur et al., 2013; Nair et al., 2014; Yamazaki et al., 2016). Signaling pathway activation as result of genomic alterations such as translocation, mutation, and copy number loss or gain has been studied to identify high-risk patients, methods of treatment, and optimal dosing (Majewski and Bernards, 2011; Wagner and Srivastava, 2012). In addition to the traditional 3+3 design (Le Tourneau et al., 2009), using adaptive clinical trial designs, biomarkers have been applied to heterogeneous patient groups that respond differently to chemotherapy (Wason et al., 2014). The combination of genomic testing and biomarkers in personalized medicine clinical research has been able to lead to comparative studies as well as randomized clinical trials (RCTs) to validate the best choice of cancer therapy for a given group of patients (Ginsburg and Kuderer, 2012). Although biomarker use in pediatric therapy lags behind that in adults, biomarker applications have been extended into the study of pharmacogenetics and pharmacogenomics of childhood cancer (Piana et al., 2012). In addition, pragmatic reasons, including limited sample volume, have restricted the ability of pediatric oncologists to routinely perform testing to support drug selection and dosing regimen. For example, minimal risk is associated with blood draw limits ranging from 1 to 5% of total blood volume (TBV) over 24 hours and up to 10% of TBV over 8 weeks (Howie, 2011; Veal, 2014), but these volumes are very small in

neonates. However, due to the rapid advancements in the sensitivity of bioanalytical assays, the above hurdle is being resolved. Table 2.3 shows representative biomarkers currently utilized in clinical oncology.

During recent years, about 20 types of tumor biomarkers have been used in the clinical arena for screening, prognosis, and targeted therapy (Guo et al., 2015). Over the last decade, it has been demonstrated that early detection of specific markers or metabolic phenotypes associated with cancer, using preferably noninvasive or minimally invasive methods, could be tremendously valuable in a clinical setting. In particular, serum biomarkers for early detection of therapeutic efficacy in cancer treatment have been a valuable routine tool in clinical studies (Linder and Alaiya, 2009). Other biomarkers for postoperative surveillance such as CA 15-3, CA 125, S100, and CA 19-9 for breast, ovarian, melanoma, and pancreatic cancers, respectively, have led to promising results (Duffy, 2013).

For example, studies have shown the utility of viable circulating tumor cell (CTC) counts as a prognostic tool in the diagnosis of epithelial tumors such as in lung, breast, colon, and prostate cancer (Pantel and Alix-Panabieres, 2016). CTC detection approaches have included IHC, reverse transcription-polymerase chain reaction (RT-PCR), and a USFDA-approved technique based on an immunomagnetic epithelial cell adhesion molecule (EpCAM) assay (Muller et al., 2012; Huang et al., 2015). For CTC detection in the peripheral blood, antibody-based enrichment, sorting by physical properties, and cell function can aid to attain better sensitivities.

Recently, a fast-growing and exciting area of cancer treatment has been the use of immunotherapy yielding an innovative class of therapeutics such as pembrolizumab, ipilimumab, and nivolumab. In this approach, the programmed death 1 (PD-1) receptor, which is a checkpoint protein on T cells, serves as an “off-switch” that prevents the T cells from attacking other cells in the body by binding with its ligand, PD-L1. Interestingly, tumors evade immune surveillance by maintaining the resting state of T cells and preventing them to be activated into an effector state. Inhibition of the so-called immune checkpoints, PD-1, PD-L1, or cytotoxic T lymphocyte antigen-4 (CTLA-4) has shown to be an effective approach in improving activation and effector function of tumor-specific T cells. For instance, several serum biomarkers such as lactate dehydrogenase (LDH), CRP, vascular endothelial growth factor (VEGF), regulatory T cells ( $T_{reg}$ ), neoantigens, and soluble CD25 have been measured during clinical trials of ipilimumab, which targets CTLA-4 (Yuan et al., 2016). However, albeit with some variability, one of the more promising biomarkers has been PD-L1 expression levels due to its

**Table 2.3** Selected examples of biomarkers in oncology research.<sup>a</sup>

Biomarker(s)	Drug	Therapeutic area
BCR-ABL, c-Kit	Imatinib, nilotinib	Chronic myeloid leukemia
VEGF, PIGF	Ramucirumab	Hepatocellular carcinoma
PD-L1, PD-L2	Nivolumab	Hodgkin's lymphoma
EGFR-positive biopsy	Cetuximab, panitumumab	Colorectal cancer
BRAF V600E mutation positive biopsy	Vemurafenib, dabrafenib	Melanoma
ERBB2 (Her2) IHC and FISH tests	Trastuzumab, pertuzumab	Breast cancer
KRAS	Cetuximab, panitumumab	Colorectal tumors
UGT1A1	Irinotecan	Metastatic cancer of the colon or rectum
UGT1A1	Nilotinib	Chronic myelogenous leukemia
Estrogen alpha expression	Tamoxifen	Breast cancer
IL2RA	Denileukin diftitox	Cutaneous T-cell lymphoma
MS4A1	Rituximab	Non-Hodgkin's lymphoma
ERBB2 (Her2) IHC and FISH tests	Ado-trastuzumab emtansine	Metastatic breast cancer
G6PD, CYB5R1-4	Rasburicase	Treatment of tumor lysis syndrome
O <sup>6</sup> -Methylguanine-DNA methyltransferase (MGMT)	Temozolomide	Glioblastoma
PI3K	Idelalisib	Chronic lymphocytic leukemia
ALK	Ceritinib	Non-small cell lung cancer

<sup>a</sup> Adapted from Wang et al. (2011), Simon and Roychowdhury (2013), Salter and Holland (2014), Zhao et al. (2015), and Jameson and Longo (2015).

upregulation in some solid tumors. Conversely, the PD-1 expression levels have been less predictable for patient stratification (Merelli et al., 2014; Meng et al., 2015; Schumacher and Schreiber, 2015; Zou et al., 2016).

Recently, research interest in a series of serum efficacy biomarkers has been reported (Linder and Alaiya, 2009). These include apoptosis markers such as Fas ligand, circulating markers of the immune system, and/or anticancer drug-mediated apoptosis including Bcl-2, caspase-1, cytochrome c, cytokeratins (CKs), and E-cadherin. In the clinical arena, the proposed circulating biomarkers can be measured in real time during the course of chemotherapy on-site using point-of-care (POC) kits or sent to off-site clinical chemistry laboratories for more elaborate testing. Continued research has also been directed to more lethal and difficult-to-treat cases such as pancreatic cancer and glioblastoma. For example, pancreatic ductal adenocarcinoma or pancreatic cancer often is characterized with poor diagnosis due to high tumor grade, low neoplastic cellularity, and lymph node properties (Corbo et al., 2012). The most common clinical pancreatic cancer marker has been elevated blood levels of CA19-9, or sialylated Lewis (a) blood group antigen, which has shown about 80% sensitivity (Corbo et al., 2012). MicroRNAs (miRNAs) in the blood have been proposed as prognostic markers for pancreatic cancer,

in particular for early diagnosis (Schultz et al., 2014). MicroRNAs are noncoding components of RNA, ranging from 18 to 25 nucleotides that play an important role in gene regulations (Kwan et al., 2016). The ultimate objective is to develop specific patterns of expression for miRNAs upon their dysregulation due to cancer. Matrices for miRNA analysis include tumor tissues such as formalin-fixed, paraffin-embedded tissue (FFPET) and whole blood using techniques such as TaqMan PCR or fluorescently tagged miRNAs (Kwan et al., 2016). Similarly, discovery of early diagnostic markers for the lethal (median survival below 15 months) malignant brain tumors or glioblastoma has been of growing interest. One such marker has been O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) promoter methylation, which is a DNA repair enzyme and measured in plasma and tissues (Tabouret et al., 2014). Several other investigational glioblastoma markers such as urine matrix metallopeptidase 9 (MMP9), miRNAs, tumor AKT, blood IGF binding protein 5, and tumor p70s6 kinase have shown promising results (Tabouret et al., 2014).

While colonoscopy has been the gold standard for colorectal cancer (CRC) screening, the method is costly, requires pre-procedure preparatory steps, and suffers from low global participation rates (Ganepola et al., 2014). Alternatively, clinical research has been directed

to discover and validate blood base markers for identification of high-risk populations. The potential CRC markers include a wide range of circulating miRNAs, aberrant DNA methylation such as in the case of early phase of tumorigenesis, tumor DNA mutation markers, and long noncoding RNAs (lncRNAs) (Ganepola et al., 2014).

## 2.9 Biomarker Measurements and Regulatory Considerations

In recent years, several regulatory guidance documents and manuscripts have been published in an attempt to harmonize the process of new biomarker qualifications and use in clinical trials (Colburn, 2003; Lee et al., 2006; Dancey et al., 2010; Fleming and Powers, 2012; Amur et al., 2015; Lavezzari and Womack, 2016). For example, there are two pathways that are accepted by the USFDA to utilize a biomarker (Amur et al., 2015). One pathway is to engage the USFDA during the drug development process by pre-investigational new drug (IND) interactions for a specific drug and continue these interactions through NDA or BLA filing. Clearly, the above approach will not apply to other investigational drugs and leaves the burden with only one sponsor. In addition, confidential sponsor–FDA discussions will not be subjected to a broad scientific review and input by other organizations. The alternative and a more recent pathway is FDA's Biomarker Qualification Program (BQP), which is consortium based and can apply to a broad therapeutic area by widespread adoption (Woodcock et al., 2011). The objective of the latter option is to make use of the qualified biomarker accessible to multiple programs by fostering industry–government–academic collaborations. Briefly, the BQP is initiated with submission of a letter of intent (LOI) by the sponsor to the USFDA. At this stage an intended context of use (COU) of the putative biomarker is defined including the establishment of analytically validated assay(s). Upon the consultation and advice stage with the FDA, a full submission package is reviewed by the biomarker qualification review team (BQRT), recommendations made, and a draft guidance posted in the Federal Register for public comment. Once public comments are considered and appropriate revisions made, the final qualification guidance is issued.

Arguably, biomarker biological qualification and its analytical assay robustness are among the key components of a successful submission package. The analytical assay falls into two general categories including (i) on-site rapid assays close to the site of patient care, which is also referred to as POC diagnostics (Rusling et al., 2010; Chan et al., 2013), or (ii) IVDs or companion diagnostics (CDx), which could involve more complex

sample processing and involvement of experienced clinical chemists.

An ideal POC diagnostic should be easier to manufacture, inexpensive, easy to use, portable, stable under certain stressed conditions, and amenable to emerging markets, uses small sample volumes, and is relatively fast. The growth of POC diagnostics has been partly due to the global challenges with cardiovascular and infectious diseases. A number of POC tests for CVDs are now commercially available and include lateral flow immunoassays with fluorescence or electrochemical detections using blood sampling (Chan et al., 2013). Likewise, the USFDA has approved a number of POC tests for human immunodeficiency virus (HIV) that often utilize gp41 and gp120 as capture peptides, are lateral flow immunoassays, and use oral fluids, serum, plasma, or whole blood (Chan et al., 2013).

Similarly, the development path to an IVD or a CDx is often parallel to a specific drug development and has the potential to facilitate its regulatory review (Drucker and Krapfenbauer, 2013). Here again, the ideal IVD should be easy to use, offers rapid turnaround, uses minimally non-invasive sampling, is cost effective, and has minimal variability (Halim, 2015). Current routes for market approval of the above devices vary among regulatory agencies (Schuh, 2008; Liotta and Petricoin, 2012; Nagai et al., 2016). For example, in the United States, medical devices are categorized into classes I, II, and III, in increasing order of the potential risk for harm to the patient. Class I devices (minimal potential for harm) are only subject to general controls and often need to be “cleared” through a 510(k) premarket notification process. Class II devices are held at a higher level of scrutiny than class I; are subject to labeling, performance standards, and post-marketing surveillance; and often require a 510(k) premarket notification. Lastly, class III devices, which constitute <10% of the overall submissions (with class II being the major component of filings), are of highest risk, life sustaining, and integral in preventing impairment of human health and/or present a potential unreasonable risk of illness or injury. Hence, class III devices often require premarket approval application (PMA) and are a major investment by a device applicant. There is also a subset of IVDs referred to as laboratory developed tests (LDTs), which are intended for clinical application, production, and specific use within a single laboratory. LDTs are developed for in-house use as a test or a service, also referred to as “home brew,” and are operated under the Clinical Laboratory Improvement Amendments (CLIA) of 1988 regulations. While categorization of devices is within the scope of the USFDA, the regulation of a CLIA lab is the responsibility of the Centers for Medicare and Medicaid Services (CMS). Table 2.4 shows examples of the USFDA-approved protein clinical biomarkers

**Table 2.4** Examples of USFDA-approved protein clinical biomarkers.<sup>a</sup>

Biomarker	Application	Sample	Assay	Submission type/device class
CA19-9	Pancreatic	Serum, plasma	Immunoassay	510(k)/II
CA-125	Ovarian	Serum, plasma	Immunoassay	510(k)/II
p63 protein	Prostate	FFPET	IHC	510(k)/I
HER2/neu	Breast	FFPET	IHC	PMA
CTCs (EpCAM, CD45, cytokeratins 8, 18+, 19+)	Breast	Whole blood	Immunomagnetic capture/immunofluorescence	510(k)/II
Alpha-fetoprotein (AFP)	Testicular	Serum, plasma, amniotic fluid	Immunoassay	PMA
c-Kit	Gastrointestinal stromal tumors	FFPET	IHC	PMA
Total PSA	Prostate	Serum	Immunoassay	PMA
Human hemoglobin	Colorectal	Feces	Lateral flow immunoassay	510(k) CLIA waived/II
BRAF V600 mutation test	Melanoma	FFPET	PCR amplification	PMA
PD-L1	Non-small cell lung cancer	FFPET	IHC using monoclonal mouse anti-PD-L1, clone 22C3	PMA

<sup>a</sup> Adapted from Fuzery et al. (2013); <http://www.fda.gov/medicaldevices/productsandmedicalprocedures/invitrodiagnostics/ucm301431.htm> (Accessed on March 28, 2016).

and their respective device designations. As is the case for the diverse field of analysis, there are other more elaborate and sophisticated tools such as mass spectrometry (MS) that have been well established in small molecule qualitative and quantification measurements. However, while identification of unknown proteins using MS-based proteomics has substantially advanced, the quantification of macromolecules continues to be a work in progress (Parker and Borchers, 2014). Standardization of protein assays, availability of a suitable internal standard, reliability of using a signature peptide for back-quantification of a larger precursor protein, refinement of sample enrichment methods for detection of low copy number macromolecules, cost of analysis, method validation turnaround, ease of assay transfer across laboratories, and complexity of performing such assays such that they are limited to experienced analytical laboratories remain to be addressed. Even in the case of small molecule biomarkers, specific measures need to be taken when performing analysis of endogenous compounds due to interference and matrix effects (van de Merbel, 2008; Jones et al., 2012; Lachance and Levesque, 2014; Thway and Salimi-Moosavi, 2014). Furthermore, small molecule tissue analysis using MS-based analysis has also reached a level of maturity (Xue et al., 2012), while the same challenges on macromolecule measurements in biofluids remains and warrants additional refinements. Using an open search PubMed database and keywords of “biomarkers and mass spectrometry,” there were 164 and

1898 hits in years 2000 and 2015, respectively. Therefore, with the recent advancement in MS instrumentation, sample processing, and enrichment, the growth of MS-based methods is expected to continue.

With respect to clinical tumor tissue biopsy samples, intra-tumor heterogeneity (ITH) across malignancies due to hypoxia, normoxia, phenotypic features, and epigenetic and genetic factors creates challenges in accurate biomarker determinations. These include inter-patient tumor, intra-tumor, inter-metastatic, and intra-metastatic heterogeneities (Jamal-Hanjani et al., 2015). Therefore, sample size and location of sampling may preclude “one size fits all” for feature-based prognostic biomarkers, tumor score, or grade assignment and understanding the tumor biology. Table 2.5 shows some of the general considerations in biomarker quantification that should be considered, particularly in a clinical arena.

## 2.10 Conclusions

During the last decade, a plethora of research publications, reports, white papers, and regulatory recommendations have led to higher appreciation and progress in the field of biomarkers and practical utility to TM. The success of a biomarker qualification does not merely lie with the analytical measurement, but must include robust biological validation. Genomic, proteomic, and metabolomic markers will continue to broaden our

**Table 2.5** General considerations in biomarker quantitative measurements.

Biomarker assay challenges	
Clinical decision on statistical cutoff decision points, consistency of baseline values, scoring methods, data processing, and reporting	Assay transfer across different labs including bridging studies; assay platform standardization
Expert data interpretation	Sample chain of custody
Availability of reference standards and reagents with certificate of analysis; availability of assay positive and negative controls	Ethical, informed consent, and data protection considerations
Bioinformatics analysis	Assay dynamic range across patient subgroups and in response to different treatment designs
Specified universal lab manual or assay protocol	Sample volume such as those in pediatric studies
Proprietary versus commercial assays (e.g., kits)	Assay turnaround time
Method development, validation, and compliance; a fit-for-purpose assay is not synonymous with a validated assay	Assay cost, vendor(s), and procurement logistics
Matrix interference, surrogate matrix	Regulatory framework and international acceptance
Adherence to LDT versus IVD	Timing of specimen collection
Shared samples for multiple biomarker determinations; impact of single versus combination therapy on a biomarker	Sample heterogeneity (e.g., intra-tumor heterogeneity); fresh versus frozen tissue
Sample collection, stabilization, shipment, and biobanking standardization; long-term sample storage stability	On-site (e.g., real-time analysis) versus off-site detection; may need distinctly different detection but complementary approaches (e.g., imaging and blood sampling)
Invasive versus noninvasive sample collection	Biomarker biological qualification
Assay reimbursement by a third party such an insurance company or a government agency	Specified universal choice of specimen/matrix: blood, saliva, breast milk, tissue, hair, amniotic fluid, expelled air, urine, sputum, tears, skin, breath, peripheral blood mononuclear cells, feces, tooth, semen, etc.

understanding of disease states and improve our toolbox capability in the area of preventive medicine.

Predictive or diagnostic biomarker discovery and qualification in such areas as chronic kidney disease (CKD) (Ju et al., 2012), infectious diseases (Downes and Shah, 2012), osteoarthritis (Kraus et al., 2011), and rapid triage for brain traumatic injury (North et al., 2012) for sports organizations, civilians, and military populations are areas of interest. Another emerging concern is nonalcoholic fatty liver disease (NAFLD) with global prevalence of 25% with 7–30% of this segment developing nonalcoholic steatohepatitis (NASH), characterized by hepatocellular injury, inflammation, and fibrosis (Caligiuri et al., 2016). A clear mechanistic understanding of NASH and biomarkers for its early detection will be needed in order to manage this condition.

A report from a recent USFDA workshop indicated the desire to investigate alternative new end points for type 1 and type 2 diabetes (Kingwell, 2016). Although glycated hemoglobin (HbA1c) has been the gold standard for

clinical outcome studies, one drawback is its limited lifespan of about 3 months due to red blood cells' life cycle. Ongoing discussions are underway to identify and validate new outcome measures.

In the technology front, real-time clinical monitoring in biofluids using noninvasive methods as well as tissue samplings using minimally invasive procedures with temporal resolution is being investigated (Rogers and Boutelle, 2013; Frost and Meyerhoff, 2015). Tissue biomarker analysis using matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) as an alternative to quantitative whole-body autoradiography (QWBA) is being utilized in preclinical and clinical studies (Jones et al., 2015; Murphy et al., 2015).

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**3**

## **Current Regulatory Guidance Pertaining Biomarker Assay Establishment and Industrial Practice of Fit-for-Purpose and Tiered Approach**

*Naidong Weng*

*Bioanalytical & Pharmacokinetics, Janssen Research & Development, LLC, Spring House, PA, USA*

### **3.1 Introduction**

In the last two decades, bioanalytical laboratories around the world have increasingly been involved in delivering decision-critical data for moving forward the drug candidates along the discovery and development pipelines. More importantly data generated from bioanalytical lab have often been used for various purposes, ranging from exploratory research to important internal go-no-go decision and all the way to regulatory filings. It can be argued that contemporary bioanalysis is one of the few disciplines in the pharmaceutical industry crossing the entire drug discovery and development process. As such, bioanalysis practitioners tend to be on the more cautious/conservative sides and generally avoid risk-taking approaches, especially in the heavily regulated bioanalysis space (i.e., supporting good laboratory practice (GLP) toxicology studies and clinical trials) where regulatory guidance is followed strictly and is treated as bible. The roots of bioanalytical guidance on method validation and sample analysis dated back to 1990 from the Crystal City I meeting, where the FDA and industrial representatives met, discussed, elaborated, and drafted the first bioanalysis-specific guidance. This was followed by a series of Crystal City meetings and a revised draft FDA guidance as well as bioanalysis guidance from other regions (European Union, Japan, Brazil, and China, just to name a few). Collectively, these guidelines provide instructions and framework for bioanalysis practitioners when conducting bioanalytical validation and sample analysis. While majority of the requirements in the guidance are consistent across different regions, some differences or even contradictions are also noted. Thus and per requirement of GLP under which most of the bioanalytical laboratories operate, bioanalytical laboratories have their own tailor-made standard of operations (SOPs) to help their bioanalytical scientists understand the interpretation of the guidance (Patel et al., 2013). Depending

on the region of the bioanalytical laboratory and the type of regulatory filing each laboratory supports, some differences in those SOPs are also noticed. These differences in practices, compounded by lack of clear regulatory clarifications on some of the requirements mentioned in the guidance, for example, biomarker assay establishment, plus the conservative nature of bioanalytical community, lead to an increase in the extent of characterization of the performance of bioanalytical assays. This has led to bioanalysis being routinely conducted at a level that may only be needed for the most heavily regulated areas such as bioequivalence (BE) or equivalent pivotal studies, even for biomarker measurement.

Practically speaking, few pharmaceutical companies still conduct regulated bioanalysis internally. It is no longer viewed as cost effective and competitive to conduct large routine bioanalysis internally. First and most importantly, with many years of development, bioanalytical work outsourced to contract research organizations (CROs) globally has matured and became a sustainable mega-billion-dollar business. Since CROs support many more studies from multiple clients and their organizations tend to be larger than a typical pharma bioanalytical department, they are able to better handle the study volume in a more cost-effective manner. Human resource flow is no longer in one direction from CROs to pharmaceutical companies, and these days many of the scientists working at CROs were former colleagues from pharmaceutical companies. They brought in both technical expertise and compliance awareness. These days, many pharmaceuticals and CROs work on preferred provider setting and therefore CROs can be viewed as extended laboratories of pharmaceutical companies. Since CROs work for multiple clients, they tend to be extremely cautious about risk taking, afraid that one misstep could potentially jeopardize their relationship and reputation with the clients. As such, a

single high standard approach to demonstrate method performance and to establish a level of performance through fixed acceptance criteria used for BE studies is the most straightforward and welcomed by most of the CROs, even though these criteria may not be necessarily linked to the purpose of the data for studies other than BE. Data generated under such a system certainly can withstand any regulatory scrutiny and minimize any risks CROs and their clients are willing to take.

On the other hand, drug development is under ever-increasing pressure. Even with tremendous effort new drug approval rates remain flat in the last many years. Better and more efficient drug development procedures are being tried. In recent years, endogenous molecules such as catabolic/anabolic products, bioactive small molecules, peptides, and proteins have been increasingly explored and utilized as biomarkers to facilitate drug discovery and development processes (Colburn and Lee, 2003; de Gramont et al., 2015). Appropriate application of biomarkers' data can enable more knowledgeable predictive and reliable decision making for target and candidate selection, risk assessment, dose escalation, patient stratification, drug–drug interaction evaluation, and safety surveillance, thus reducing the attrition rate in the late-phase clinical trials and improving the productivity of bringing new therapies to patients. As a consequence, needs for the pharmaceutical industry to be more efficient in biomarker bioanalysis call for customized approaches to establishment and use of bioanalytical methods to support biomarker research at different stages of drug discovery and development. To use one-fits-all approach like bioanalytical support certainly will not sustain the economic burden and required turnaround time to support timely decision making. Indeed, as early as in 2006, the “fit-for-purpose” paper of Lee et al. already discussed a customized bioanalytical approach focusing on biomarkers (Lee et al., 2006).

In this chapter, we discuss several topics of bioanalytical method validation and sample analysis pertaining to biomarker:

- 1) Current regulatory guidance and interpretation
- 2) Current industrial discussion and recommendations
- 3) Considerations for assay validation and sample analysis
- 4) Examples of fit-for-purpose and tiered approach

### **3.2 Current Regulatory Guidance and Interpretation**

Currently, none of the regulatory guidance gives specific and detailed instruction and criteria on how to establish a bioanalytical method to measure biomarkers even

though it has been well recognized widely, including by the FDA, that lack of analytical method vigor is one of the major reasons why most of the biomarkers fail to deliver useful information in the pivotal clinical studies. The 2013 draft guidance has a section dedicated to endogenous compounds and biomarkers (USFDA, 2013. Addition Issues VI) even though neither the 2001 guidance (USFDA, 2001) nor the original 3rd American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop conference report included biomarkers in its discussion scope (Viswanathan et al., 2007): “Because of the important roles biomarkers can play in evaluating the safety and/or effectiveness of a new medical product, it is critical to ensure the integrity of the data generated by assays used to measure them. Biomarkers can be used for a wide variety of purposes during drug development; therefore, a fit-for-purpose approach should be used when evaluating the extent of method validation that is appropriate. When biomarker data will be used to support a regulatory action, such as the pivotal determination of safety and/or effectiveness or to support labeled dosing instructions, the assay should be fully validated. For assays intended to support early drug development (e.g., candidate selection, go-no-go decisions, proof-of-concept), the sponsor should incorporate the extent of method validation they deem appropriate.” It went on further to state “Method validation for biomarker assays should address the same questions as method validation for PK assays. The accuracy, precision, selectivity, range, reproducibility, and stability of a biomarker assay are important characteristics that define the method. The approach used for pharmacokinetics (PK) assays should be the starting point for validation of biomarker assays, although FDA realizes that some characteristics may not apply or that different considerations may need to be addressed” (USFDA, 2013).

The FDA appears to make distinguish between endogenous biomarkers and endogenous compounds that are therapeutics. The requirement of using authentic matrix for calibration standards for the endogenous therapeutics is not listed for the endogenous biomarkers. As we will discuss later, for biomarker quantitation, it is routinely acceptable that calibration standards can be prepared in surrogate matrix as long as appropriate quality control (QC) samples are prepared in authentic matrix. Nevertheless, the FDA stresses that the approach used for PK assays should be the starting point for validation of biomarker assays even though it is recognized that fit-for-purpose approach should be used both in this draft guidance and in a paper authored by an FDA office (Booth, 2011). A number of conference reports and consortium position papers were published focusing on how to interpret this requirement and to debate and elaborate which type of biomarker bioanalysis requires which kind

of tier or fit-for-purpose approach. A white paper was published in 2014 on the Crystal City V workshop report on quantitative bioanalytical method validation and implementation for the 2013 revised FDA guidance (Booth et al., 2015). The white paper reflects the consensus and compromise between the FDA and the pharmaceutical industry. Biomarkers are categorized into two broad categories based on the intended use of the data. Category 1 is for assays that generate data for internal decision making and do not drive label claim, while category 2 is for assays that generate data for pivotal assessment of efficacy or label dosing instruction. Biomarker assays can be further categorized into three groups—qualitative assay, relative assay, and definitive assay. For a category 2 assay, if a definitive quantitative assay can be developed, then the assay should be validated to meet the same standards of a PK assay.

In responding to the request to clarify those statements relevant to biomarker assay development and validation, the Crystal City VI workshop was organized by the AAPS in association with the US FDA to continue discussion on the bioanalysis of biomarkers and was reported in the conference report by Lowes and Ackermann (2016). It was agreed that while the need to operate within the framework of the current bioanalytical method validation guidance was clearly acknowledged, a general understanding that biomarker method validation cannot be adequately depicted by current PK-centric guidelines emerged as a consensus from the meeting. Because of inherent difficulty in achieving truly accurate measurements for endogenous molecules, it was quickly understood that most quantitative biomarker measurements will be made by using relative quantitation and not by definitive methods.

The European Medicines Agency (EMA) bioanalytical method validation guidance (July 21, 2011) states that “Methods used for determining quantitative concentrations of biomarkers used in assessing pharmacodynamics endpoints are out of the scope of this guideline.” However, the EMA also pays close attention to research on the use of biomarkers in the development of medicines. In August 2014 the EMA issued a draft concept paper outlining the key elements to be developed in a guideline on good genomics biomarker practices. A Joint FDA/EMA Letter of Intention (LOI) Submissions for Biomarkers and Clinical Outcome Assessment Qualification Programs was issued. Parallel submissions for qualification of biomarkers to both agencies are encouraged, and both FDA and EMA will share their scientific perspective, advice, and response letters for the submission. Additionally, the fit-for-purpose bioanalytical method validation concept, which is essential for biomarker assay validation, is already embedded in the EMA bioanalytical method validation guidance:

“Acceptance criteria wider than those defined in this guideline may be used in special situations. This should be prospectively defined based on the intended use of the method.” and “Methods used in pre-clinical studies not required to be performed to GLP should be fit for purpose but not necessarily developed in a GLP facility” (European Medicines Agency, 2011). It can be certainly anticipated that biomarker assay establishment could be one of the future additions for EMA bioanalytical method validation.

Similar to EMA bioanalytical method validation, biomarker assay establishment is not discussed in Japanese guideline on bioanalytical method validation in pharmaceutical development (MHLW 2013). Based on the scope of this guidance, “The information in this guideline generally applies to the quantification of low-molecular-weight drugs (except for endogenous substances), by analytical methods such as liquid chromatography (LC) and gas chromatography (GC) used in combination with mass spectrometry (MS) or with the other detectors.” It can be assumed that biomarkers (endogenous substances) are not in the scope of this guidance. The term “Application of Tiered Approach” is explicitly described in the annex of this guidance. Although it references metabolite analytes, the concept of tiered approach can be extended to state the following: “The tiered approach is a strategy to limit the characterization of an analytical method initially and to gradually expand parameters to be characterized and moving toward a full validation as the development process proceeds.” It also clearly states the requirement for conducting tiered approach: “However, even when the tiered approach is used, it is advisable to predefine appropriate criteria for the characterization of analytical method based on scientific judgment in order to improve the reproducibility and reliability of concentration data obtained.”

### 3.3 Current Industrial Discussion and Recommendations

Industrial discussion and deliberation of biomarker assay validation requirement occurred before the incorporation of biomarker assay in FDA draft guidance. Early bioanalytical practitioners of biomarker assay establishment already published a leading article and established the framework for today’s fit-for-purpose discussion (Colburn and Lee, 2003). It was already recognized that biomarker assays and resulting validation are generally more complicated than those for most drug assays, and it is generally not possible to validate biomarker analytical methods according to strict GLPs. One challenge for biomarker assays is finding analyte-free matrices to prepare calibration standards. Existence of heterogeneous *in vivo*

biomarker forms is another challenge for biomarker assay, and relative quantification rather than definitive quantification is often applied. Additional challenges for biomarker assays include the identification and selection of biomarkers in an accessible and useful target matrix as well as biological and assay method variability.

Based on the AAPS Biomarker Workshop conference report (Lee et al., 2005), in their landmark paper, Lee et al. further elaborated the concept of fit-for-purpose method development and validation for successful biomarker measurement (Lee et al., 2006). The “fit-for-purpose” approach to biomarker method development and validation is proposed to address the extent to which a biomarker assay should be validated for intended use as it is considered to provide for efficient drug development by conserving resources. The method validation processes include four activities of prevalidation (preanalytical consideration and method development), exploratory method validation, in-study method validation, and advanced method validation. The processes are continuous and iterative and driven by the intended purpose of the biomarker data. The normal flow is from biomarker development (prevalidation) to method validation (exploratory or advanced) to application (in-study method validation). The process could be moving the chosen biomarkers from mechanism exploration to pilot in-study and to advanced validation for confirmatory studies or from exploratory validation to advanced validation due to a critical business decision change.

Two years later, Chau et al. published a perspective paper on validation of analytical methods for biomarkers used in drug development (Chau et al., 2008). This paper focuses on the general principles of the biomarker validation process with an emphasis on assay validation and the collaborative efforts undertaken by various sectors to promote the standardization of this procedure for efficient biomarker development. The integration of the biomarker assay validation and qualification process with drug development was discussed extensively. Fit-for-purpose method validation principle should be applied to exploratory, pre-study, advanced, and in-study validation. In order to apply fit-for-purpose strategy, one also needs to understand the FDA biomarker qualification process and drug development process so that the appropriate level of assay validation can be executed.

In 2010, Cummings et al. further elaborated on how to conduct fit-for-purpose biomarker method validation (Cummings et al., 2010a, 2010b). These two papers attempted to provide and clarify some of the scientific and regulatory issues surrounding biomarker method validation and sample analysis from clinical trial subjects. Using specific examples, authors of this paper also strived to provide clear guidance on validation strategies for establishing definitive, relative, quasi-qualitative, and

qualitative biomarker assay performance parameters such as accuracy, bias, precision, reproducibility, sensitivity, specificity, dilution linearity, parallelism, assay range, reagent stability, and sample stability.

Due to the complexity of biomarker assay development, validation, and sample analysis, multiple consortiums and workshops were also formed with the attempt to further debate, elaborate, and provide clearer instruction to bioanalytical practitioners and to establish a frame for further discussion with regulatory authorizers around the world. Recommendations from white papers or position papers from these focus groups are in general very useful albeit some inconsistency is unavoidable. Since publications of the earlier papers from Lee, Chau, and Cummings that focused mainly on the ligand binding assays (LBA), great progress has been made in the mass spectrometry (MS) community for the use of liquid chromatography in conjunction with mass spectrometry (LC–MS). Effort has been made to provide guidance on LC–MS assay establishment for biomarker analysis.

In 2012, Global CRO Council (GCC) published a white paper on recommendations on biomarker method validation. The survey conducted by this paper indicated that in 2012 majority of the organizations apply fit-for-purpose approach to develop methods. The consensus reached was that when there is a definitive quantitative clinical end point, the biomarker assay needs to be fully validated and the acceptance criteria should be set based on assay performance and the anticipated use, while when there is a relative clinical end point, the biomarker assay could be validated using the fit-for-purpose approach although the definitions of definitive and relative clinical end points were not given and their relevance to the significance of pivotal decision making and regulatory filing or label claiming was not stated. Instead of using previously proposed four-tier method establishment (definitive, relative, quasi-qualitative, and qualitative) originally proposed by Lee et al., this white paper recommended to use three tiers—validated, qualified, and screening—with the intention of being more intuitive for bioanalytical scientists when conducting assay establishment. This paper also suggested minimum requirements for a qualified method (middle tier) with the option of setting acceptance criteria based on the performance of the assay during the qualification process. For LC–MS methods, establishment of the following assay parameters were recommended: calibration, precision and accuracy, selectivity/specificity, matrix effects/parallelism, limited storage stability, and sensitivity.

The importance of integrating all scientific aspects, from purely analytical aspects all the way to understanding the biology and effects of the biomarker, prior to embarking on method establishment or sample analysis, is emphasized by the European Bioanalysis Forum (EBF)

(Timmerman et al., 2012). Close and iterative interactions with the teams requesting the data are imperative to develop a bioanalytical strategy that combines science, analytical performance, and regulations. A decision tree for biomarker method establishment and analysis of study samples was proposed.

The three-part white papers from Workshop on Recent Issues in Bioanalysis (WRIB), which were published in 2015, focus on assay establishment for biomarkers (Part 1, small molecule LC–MS; Part 2, large molecules hybrid ligand binding assay (LBA)/LC–MS; Part 3, large molecule bioanalysis using LBA, biomarkers, and immunogenicity) (Ackermann et al., 2015; Amaravadi et al., 2015; Yang et al., 2016). Main challenges and approaches for successful development and validation of bioanalytical assays for small and large molecule endogenous biomarker using LC–MS and LBA were discussed. It was suggested that high-resolution MS (HRMS) could provide additional insurance on assay selectivity. Since the platform for hybrid LBA/LC–MS is relatively recent, many unanswered regulatory questions and technical challenges were discussed extensively.

Fit-for-purpose bioanalytical assay establishment was also extensively discussed by the Global Bioanalysis Consortium (GBC) (Lowes et al., 2015). This commentary paper does not address biomarker assays specifically, although again the principles can be taken into consideration for addressing the fit-for-purpose needs of such bioanalytical methods.

Several editorial, commentary, or focus papers in the journal of *Bioanalysis* also attempted to provide additional insights on fit-for-purpose biomarker assay approaches (Houghton and Chamberlain, 2011; Houghton et al., 2012; Valeri et al., 2013; Timmerman, 2014; Arnold et al., 2016; Bennett, 2016; Cowan, 2016; Timmerman, 2016). All these papers, from similar or different angles, acknowledge the challenges of establishment of biomarker assays and the notion of fit-for-purpose and tiered approach for biomarker assay development.

Important considerations for quantitation of biomarkers using LC–MS were discussed in an editorial paper from our laboratory (Jian et al., 2012). The endogeneity of biomarkers, and often the exploratory nature of the studies, presents a series of challenges to bioanalytical scientists. In our opinion, dealing with endogenous levels of the analyte, confirming specificity of the assay, maintaining analyte integrity ex vivo, and designing the proper study protocol are the most important aspects of successful biomarker assay development. Challenge of assay specificity owing to the endogenous nature of biomarker was discussed extensively and strategy of confirming assay specificity was proposed. Interference from isobaric compounds or structural analogs that may be generated from the same biological

pathway as the targeted analytes is particularly prevailing. Endogenous biomarkers may have many isobaric positional isomers generated from enzymatic or chemical reactions existing in the biological matrix, which share the same multiple reaction monitoring (MRM) transitions as the analyte in MS detection, therefore causing specificity problems. Different strategies can be attempted to confirm the specificity of an LC–MS assay including comparison of retention time, instrument response, or shape of the targeted chromatographic peak, the product ion or MS<sup>3</sup> spectra, and the signal intensity acquired under alternative MRMs between standard in neat solution and standard in extracted sample. HRMS data can be also used to help confirm the assay specificity. Another important area for consideration that is often overlooked is the study design. Bioanalytical support for a biomarker study goes beyond evaluation of a series of bioanalytical parameters. To answer the fundamental question about changes in biomarker levels as a result of drug treatment, toxicity, or disease condition, it is essential to have knowledge about their naturally occurring variations, such as those caused by interindividual differences, diurnal changes, and food effects.

Since tiered and fit-for-purpose approaches are often used for biomarker assay development, there is a misconception among some of the bioanalytical practitioners that low tiered biomarker assays can have low and sloppy quality. It is worthwhile to clarify here that in my opinion low tier is not a problem and it is acceptable, while a low quality is always a problem and is never acceptable. Quality is about keeping your promise that the assay will perform with a certain behavior, and tier is a category of the assay. Tiered approach should be part of the integrated planning and should not be an afterthought. Tiered approach stresses the scientific understanding of the issues and takes appropriate actions. Tiered approach is not a “get out of jail” card and you will need to have organizational system to ensure the quality. Data generated from tiered and fit-for-purpose approach methodology should still maintain data integrity and reconstructability. Lastly tiered and fit-for-purpose approach, when used appropriately, can be acceptable to health authorities for biomarker research.

### 3.4 Considerations for Assay Validation and Sample Analysis

As fit-for-purpose biomarker assay establishment is highly complicated, the following parameters and recommendations for method development and validation considerations are largely driven by scientific consideration.

### 3.4.1 Sensitivity

A low limit of quantitation (LLOQ) should be appropriate for applications and should be able to capture the change of biomarker levels at both basal levels and upon intervention. Typically, an LLOQ at 50% lower than the lowest basal value would be sufficient to capture both basal and elevated levels. One would need to adjust the LLOQ if the anticipated change upon intervention is the decrease of the biomarker level. Since biomarkers are endogenous compounds, in many cases it is a challenge to assess the LLOQ in the authentic matrix using authentic analyte. Strategy of mitigating this includes use of a surrogate analyte (usually a stable labeled analyte) and a surrogate matrix. In order to obtain correct reading of LLOQ, parallelism or equivalency of LC-MS responses of the authentic and surrogate analytes should be considered for surrogate analyte approach, and matrix effects difference should be considered for the surrogate matrix approach.

### 3.4.2 Specificity and Selectivity

For the determination of endogenous substances, the specificity of the assay, or unambiguous identification of the peaks of interest, is a critical part of method development. This can be achieved by a chromatographic retention and mass spectrum match with a well-characterized reference standard. During method development, if the laboratory has access to an HRMS, then additional identification efforts should be considered to ensure peak purity (Jian et al., 2012).

In order to avoid ionization suppression due to other endogenous/exogenous compounds as well as chromatographic resolution between biomarkers of interest and other isobaric compounds, chromatographic run time for biomarker assays is often significantly longer than LC-MS assays for drugs and metabolites. For example, it is recommended that complete chromatographic resolution be obtained between 4 $\beta$ -hydroxycholesterol, a potential endogenous biomarker for CYP3A4 induction, and cholesterol, which is typically present at over 10,000-fold higher concentration than 4 $\beta$ -hydroxycholesterol (Aubry et al., 2016). Co-elution of cholesterol with 4 $\beta$ -hydroxycholesterol could lead to severe ion suppression of 4 $\beta$ -hydroxycholesterol. Another critical selectivity test during the development of a 4 $\beta$ -hydroxycholesterol assay is to obtain sufficient chromatographic resolution between the 4 $\alpha$  and 4 $\beta$  isomers, since they have similar fragmentation and mass spectrometric responses. In addition to 4 $\alpha$ -hydroxycholesterol, it is recommended to check for interferences from other oxidative metabolites of cholesterol, either with authentic substances, for those

compounds that are available commercially, or by evaluating several sources of human plasma.

### 3.4.3 Matrix Effects and Sample Variables

Three different analytical approaches have been used successfully in the determination of biomarkers in biological fluids. All can be validated for the intended applications. The procedures used to estimate matrix effects (for LC-MS assays) and matrix-lot variability in each approach are described later.

#### 3.4.3.1 Authentic Analyte/Authentic Matrix Approach

If the biomarker basal level is very low and is elevated significantly upon intervention, the same and well-established approach used for PK assays can also be applicable (Lin et al., 2013). Matrix effects are established in the same manner used for PK assays, by comparing the analyte response in solvent and post-spiked in extracted blank matrices. It is recommended to use matrix from at least six individual donors and evaluate whether matrix effects are different.

#### 3.4.3.2 Surrogate Analyte/Authentic Matrix Approach

In this approach, the reference standard is a stable labeled analog of the endogenous analyte. Matrix effects are established in the same manner used for authentic analyte/authentic matrix, by comparing the analyte response in solvent and post-spiked in extracted blank matrices.

#### 3.4.3.3 Authentic Analyte/Surrogate Matrix Approach

In this approach, the reference standard is analyte and a surrogate matrix containing no analyte is used to prepare the calibration standards. The absolute matrix effect cannot be measured directly. Relative matrix effects are estimated by testing the apparent reproducibility of the measured concentration of the analyte spiked at one or more concentrations in multiple lots of authentic matrix. For this experiment, it may be advantageous to use pre-screened matrix lots with low endogenous levels of the biomarker analyte.

### 3.4.4 Accuracy/Precision

In general, the accuracy for a biomarker assay is not as critical as for a bioanalytical assay designed for PK evaluation (Lee et al., 2006). Biomarker assays usually call for a comparison of pre- and posttreatment levels. On the other hand, precision is critical to the validity of the data for decision making.

### 3.4.5 Stability

Fit-for-purpose stability assessment should be performed. In general, stability assessments including stability in whole blood, plasma/surrogate matrix for benchtop, freeze/thaw, long-term storage, processed sample, autosampler stability, and solution stability should be included as needed. Regardless of surrogate or authentic matrix, biological stability needs to be established using authentic analyte in authentic matrix. Based on the nature of the biomarker assay, additional stability assessment might also be needed. For example, one concern for stability of the 4 $\beta$ -hydroxycholesterol assay is related to cholesterol auto-oxidation to 4 $\beta$ - and 4 $\alpha$ -hydroxycholesterol. It is therefore important to assess the effectiveness of any measure implemented to prevent this type of oxidation (e.g., addition of antioxidants). Excessively high levels of 4 $\alpha$ -hydroxycholesterol may indicate compromised sample stability (Aubry et al., 2016).

It should be noted that a surrogate analyte may demonstrate a different stability profile from the corresponding authentic analyte. For example, if there is ex vivo biosynthesis/conversion to generate the endogenous analyte, it cannot be elucidated by just monitoring a surrogate analyte because there is no such equivalent process for these exogenous molecules. This has been observed in our work on quantitation of fatty acid amides (Jian et al., 2010). The endogenous analytes showed rapid ex vivo elevation in whole blood over the time, which was absent for the surrogates. This was because the endogenous analytes were being continually released from their precursors in blood cells, leading to an increase in the measured concentrations.

Therefore, no matter which approach is taken for biomarker bioanalysis, it is always crucial to evaluate stability of the authentic analyte in authentic matrix, including the long-term storage stability, either preexisting or post-spiked, to elucidate if there is any instability loss or in some cases ex vivo biosynthesis of the analyte.

### 3.4.6 Sample Analysis Consideration

During sample analysis, an important factor is to monitor assay performance, including the chromatographic resolution between the biomarker and its interference compounds. It might be appropriate to establish these separations by injecting a system suitability solution if the separation cannot be easily assessed from QC or incurred samples.

For assays that are used to assess small to moderate biomarker level changes, it is recommended to analyze the pre-dose and post-dose samples within the same run to minimize assay variability. This is similar to the approach used commonly in BE studies and will maximize the utility of the assay.

## 3.5 Examples of Fit-for-Purpose and Tiered Approach

Fit-for-purpose and tiered approach biomarker assay development and validation should always be used in conjunction with the purpose of the study. The following are some examples of its applications.

### 3.5.1 Relative Quantification of Glyco-isoforms of Intact Apolipoprotein C3 in Human Plasma by LC-HRMS

Glycosylation is one of the most important posttranslational modifications to mammalian proteins. Distribution of different glyco-isoforms of certain proteins may reflect disease conditions and therefore can be utilized as biomarkers. Apolipoprotein C3 (ApoC3) is among one of the plasma glycoproteins extensively studied for its associations with disease. ApoC3 mainly exist in three glyco-isoforms, including ApoC3-1 and ApoC3-2, which contain O-linked carbohydrate moiety consisting of three and four molecules of sugars, respectively, and ApoC3-0, which lacks the entire glycosylation chain. Changes in the ratio of different glyco-isoforms of ApoC3 have been observed in pathological conditions such as kidney disease, liver diseases, and diabetes. A relative quantitation approach of measuring the concentration ratios of the three glyco-isoforms using LC–MS was developed (Jian et al., 2013a). The fit-for-purpose assay validation for relative quantitation includes the following assay parameters: precision (reproducibility) and stability (benchtop; freeze/thaw; long-term storage).

### 3.5.2 Quantification of 4 $\beta$ -Hydroxycholesterol Endogenous Biomarker for CYP3A4 Activity in Plasma Samples

Endogenous CYP3A4 biomarker 4 $\beta$ -hydroxycholesterol shows moderate increase upon dosing strong CYP3A4 inducer rifampin and currently is proposed as a biomarker to evaluate potential drug–drug interactions for CYP3A4 induction of the drug candidates. Typically, about four- to eightfold increases in 4 $\beta$ -hydroxycholesterol for strong CYP3A4 inducers are observed and statistical significance from baseline reached by day 3 post-dosing with rifampicin (600 mg QD). On the other hand, with the dose of a strong CYP3A4 inhibitor, ketoconazole, only ~20–40% yet robust decrease of 4 $\beta$ -hydroxycholesterol was observed (Kasichayanula et al., 2014). Statistical significance from baseline is reached by day 3 post-dosing with ketoconazole (400 mg QD). Currently 4 $\beta$ -hydroxycholesterol has not been recommended as a probe for

the CYP3A4 inhibition due to the fact that this level of biological changes could easily fall within the perceived assay variability (Aubry et al., 2016). However, a more precise biomarker assay (e.g., 5% CV instead of current typical 15–20% CV) would certainly make this important biomarker useful to assess CYP3A4 inhibition. This is a good example that fit-for-purpose biomarker assay development should not always lead to a lower tier assay.

### 3.5.3 Quantitation of Leukotriene B4 in Human Sputum as a Biomarker Using UPLC-MS/MS

Leukotrienes (LTs) are a group of potent proinflammatory mediators that are enzymatically generated from their precursor, arachidonic acid. Initially, we attempted to analyze LTB<sub>4</sub> along with cysteinyl leukotrienes LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> (Jian et al., 2013b). However, LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> all experienced significant losses in both non-dithiothreitol (DTT)-treated (used as a liquefaction reagent for the sputum) and DTT-treated sputum, and LTB<sub>4</sub> is only stable in non-DTT-treated sputum. It was therefore for the purpose of current study that measurement of LTB<sub>4</sub> was sufficed. A fit-for-purpose strategy for

method development, assay qualification, and study support was adopted for this biomarker project. Due to limited supply of authentic matrix, a surrogate matrix (protein buffer) was used for the preparation of calibration samples and certain levels of QC samples to avoid interference from an endogenous analyte, while the low QC was prepared in an authentic matrix, human sputum. Chromatographic separation of LTB<sub>4</sub> from its three nonenzymatically derived isomers, that is, 6-*trans*-LTB<sub>4</sub>, 12-epi-LTB<sub>4</sub>, and 6-*trans*-12-epi-LTB<sub>4</sub>, was achieved.

## 3.6 Conclusion

Biomarker assay establishment is complicated and fit-for-purpose assay development requires the bioanalytical practitioner to fully understand the aim of the study and current regulatory requirement as well as industrial best practices. Fit-for-purpose biomarker assay development should be scientifically driven and carefully strategized early on in the drug program and frequently revisited and refined.

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**4**

## **Modern Liquid Chromatography and Mass Spectrometry for Targeted Biomarker Quantitation**

*Wenying Jian*

*Bioanalytical & Pharmacokinetics, Janssen Research & Development, LLC, Spring House, PA, USA*

### **4.1 Introduction**

In clinical chemistry and drug discovery and development, various biomarkers including small and large molecules are quantified using a variety of techniques and instrumentalities. In the past years and even now, research use-only commercial kits and FDA-approved diagnostic kits have been the major platforms for the targeted quantitation of biomarkers. These ligand binding-based assays provide good sensitivity and high throughput capability but often have the disadvantage of lacking specificity. Cross-reactivity from structurally similar molecules and interference caused by other endogenous or exogenous components in biological samples could compromise the accuracy and precision of the measurement. This is particularly prominent for biomarkers due to their endogenous nature, by which many highly structurally similar analogs could be derived in the body from the same biological pathway that generates the targeted analytes. Examples are the structurally closely related eicosanoids, steroid, biogenic amines, and so on (Mesaros et al., 2009; de Jong et al., 2011; Settlage et al., 2015). Quantitation of peptides and protein biomarkers could be complicated by existence of different forms such as pro-form and active form, as well as posttranslational modifications (Ackermann et al., 2015). Due to these reasons, LC–MS-based techniques, owing to their ability to provide highly specific detection, have gained significant popularity in recent years and become one of the major platforms for quantitation of biomarkers in biological samples (Chappell et al., 2014; Amaravadi et al., 2015). LC–MS has been extensively utilized in “-omics” types of work, such as proteomics, metabolomics, lipidomics, and glycomics research, for profiling of large number of unknown analytes for identification of useful biomarkers. Another important area of application is targeted quantitation, which is a more specific analysis of a known analyte as biomarker. Even though both

approaches use LC–MS, significant difference exists in many aspects such as instrument type, instrument setting, sample preparation, data acquisition mode, data analysis approaches, and so on. This chapter focuses on the basic concept and application of major LC–MS instruments used for targeted quantitation.

LC–MS has unique advantages for targeted quantitation over other techniques such as ligand binding assays (LBA). It has intrinsic selectivity based on unique properties of the analyte such as chromatographic behavior, molecular weight, and fragmentation pattern in mass spectrometry (MS). Closely related structural analog or even isomers can be differentiated by LC–MS. The possibility of using internal standard can compensate many variables during the sample processing and analysis and significantly boost the performance of the assay. Modern MS due to their fast scan speed can monitor multiple analytes at the same time, which significantly improves assay efficiency. Furthermore, data acquired by nontargeted scan mode on high-resolution MS (HRMS) can be not only processed for quantitation purpose but also explored to elucidate potential interesting structural information. Of course, LC–MS has its limitation such as generally lower sensitivity than LBA, particularly for large molecule analysis, high instrument cost and maintenance, and the need of experience and skills to operate complicated instrumentalities. Nevertheless, LC–MS plays important roles in biomarker research and will continue to improve as the technique evolves.

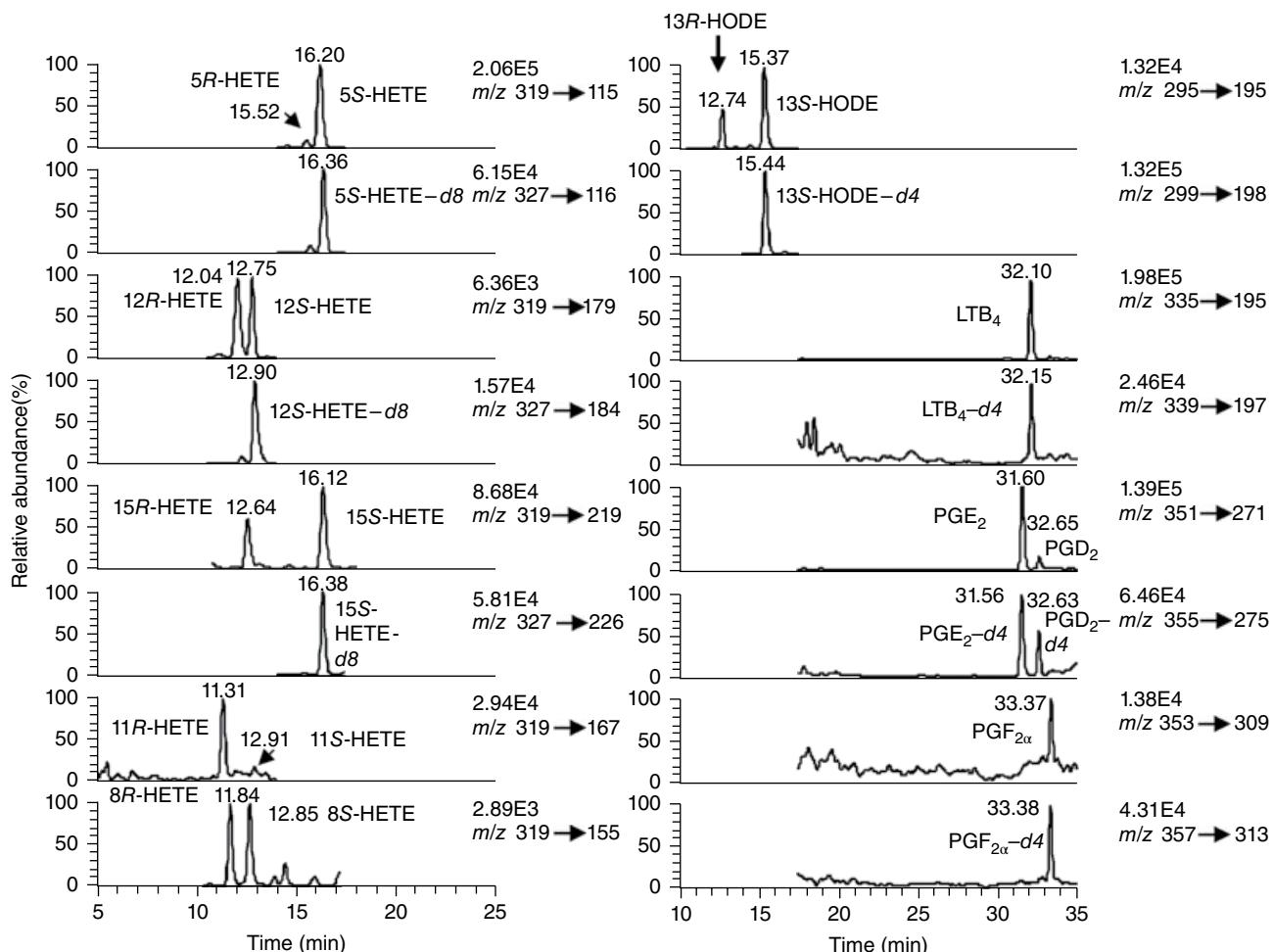
### **4.2 Liquid Chromatography**

#### **4.2.1 Importance of Separation**

For targeted LC–MS analysis, multiple reaction monitoring (MRM) on triple quadrupole instrument often provides highly specific detection of the analyte. There have been arguments on whether or not LC separation is

still necessary. In many cases, when the sample matrix is relatively simple and clean, LC separation has been actually removed from the system. For example, the RapidFire system, which employs an ultrafast online extraction system without LC column, can achieve 8–10 seconds/sample and has been extensively used in drug discovery research for in vitro functional biochemistry assays and high-throughput absorption, distribution, metabolism, and excretion (ADME) screening assay (Jian et al., 2011; Wagner et al., 2011). However, for biomarker quantitation, LC separation should still be considered to be essential. First of all, biomarker quantitation often involved complex biological matrix such as plasma, blood, tissue, and cerebrospinal fluid (CSF), which contains large amounts of endogenous components. Salts, proteins, fatty acids, and phospholipids may cause suppression or enhancement of ionization in the LC–MS interface, compromising the assay performance (Chambers et al.,

2007). More importantly, biomarkers themselves are endogenous molecules. The biological pathways that generate the biomarker of interest often produce many of its analogs either as up- or downstream products or as by-products. For example, the eicosanoid pathways can either enzymatically or nonenzymatically generate a series of arachidonic acid derivatives that are structurally closely related, most famous of which are prostaglandins. In the example shown in Figure 4.1, different positional isomers and enantiomers generated from fatty acid by lipoxygenase, cyclooxygenase, and reactive oxygen species upon stimulation of the cells can be specifically detected and quantified within single LC–MS run, based on superior separation power of the normal-phase chiral separation employed in this study (Jian et al., 2009). Another example is analysis of 4 $\beta$ -hydroxycholesterol (4 $\beta$ -OHC), which is an emerging biomarker for CYP3A4/5 induction (Kasichayanula



**Figure 4.1** Chromatograms of targeted analysis of lipid metabolites from cultured cells using LC–MS/MS analysis. HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; LTB, leukotriene; PG, prostaglandin. Source: Jian et al. (2009), <http://www.jbc.org/content/284/25/16799.short>. Used under CC BY 4.0 <https://creativecommons.org/licenses/by/4.0/>.

et al., 2014). To achieve accurate quantitation of  $4\beta$ -OHC, its isomers, particularly  $4\alpha$ -hydroxycholesterol ( $4\alpha$ -OHC) generated by auto-oxidation, has to be chromatographically separated (van de Merbel et al., 2011).

#### 4.2.2 Basic Principle of LC

LC separates compounds based on their characteristics such as hydrophobicity, polarity, charge status, molecule size, and so on and their interactions with stationary phase and mobile phase. In practice, separation efficiency of a column can be measured by plate number ( $N$ ), which is often calculated by a formula based on retention time and peak width. Alternatively, peak capacity ( $nc$ ) is often used to express chromatographic efficiency, which is defined as the maximum number of peaks that can fit side by side between the first (or unretained peak) and the peak of interest, with unit resolution (Francois et al., 2009). Under a given flow rate, chromatographic systems with higher efficiency provide sharper peaks than those with lower column efficiency. Another important term is selectivity ( $\alpha$ ), which refers to the capacity of a chromatographic system to retain analytes of interest to a significantly greater extent than others, without taking consideration of the width of the peaks. Sufficient selectivity does not guarantee good efficiency as two well-separated peaks may have very broad peak shape, and vice versa, as two sharp peaks may not be fully resolved. Provided acceptable column efficiency and selectivity have been achieved, attention has to be paid to achieve sufficient retention factor ( $k'$ ), a parameter reflecting extent of retention of the analyte on the column. Retention factor  $k'$  is calculated by  $(t' - t_0)/t_0$ , where  $t'$  is the retention time of the peak of interest and  $t_0$  is the retention time of an unretained compound. A retention capacity of three to five is generally considered sufficient for the purpose of quantitative bioanalysis using LC–MS because the mass spectrometer can further differentiate co-eluting components by their unique molecular weight and fragmentation. In targeted bioanalysis of biomarkers, higher retention factors may often be needed in order to achieve separation from potential structurally related endogenous analogs and other interferences. Retention capacity can be improved by altering stationary phase, mobile phase composition, and column temperature.

#### 4.2.3 Major Modes of LC Used for Targeted Biomarker Quantitation

Reversed-phase liquid chromatography (RPLC) is the most frequently used LC method for separation of small molecule biomarkers, as well as peptides released by proteolytic digestion for bottom-up quantitation of protein biomarkers. On RPLC, the analytes are retained by

their hydrophobic interaction with the nonpolar stationary phase. The most commonly used stationary phase is silica treated with organosilane [ $R(CH_3)_2SiCl$ ], where  $R$  represents different bonded phases (e.g. C18, phenyl) to provide specific selectivity. Due to steric hindrance, a rather large number of unreacted silanol groups remain underivatized on the surface of silica and could mediate secondary interactions with analytes. Endcapping processes using a very small organosilane reagent that can reach the surface and react with the remaining silanols are employed to further reduce the number of free silanol groups and therefore the secondary interactions. Another type of RPLC stationary phase is polymer based, such as polystyrene divinylbenzene (PSDVB). The polymeric materials allow better column stability under a wider range of pH and higher temperatures than silica-based columns, but may provide lower column efficiency than C18 columns. Commonly used mobile phase for RPLC is any miscible combination of water as a weaker solvent and various organic liquids such as acetonitrile and methanol as stronger solvents. Buffers or additives such as ammonium acetate or formic acid are often added to the mobile phase to modify the ionic strength and pH, which may have a significant impact on the retention, selectivity, and sensitivity of ionizable analytes. Unlike those used in other modes of detection such as LC–UV (e.g., dipotassium phosphate, sodium hydroxide), only volatile buffers or additives such as ammonium acetate and formic acid can be used for LC–MS application because nonvolatile ones are not compatible with the MS ion source.

It is very challenging to retain polar compounds such as nucleotides, amino acids, and peptides under traditional RPLC conditions, because the retention is based on hydrophobicity interaction. To overcome this issue, ion-pairing chromatography has been explored as an effective alternative. The ion-pairing reagents are added into the mobile phase to form intrinsic ion pairs with the analytes, thus reducing the polarity and improving their chromatographic retention. Triethylamine (TEA), dibutylamine, and perfluorinated carboxylic acids with  $n$ -alkyl chains such as trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), nonafluoropentanoic acid (NFPA), and so on are the commonly used volatile ion-pairing reagents for LC–MS analysis (Gao et al., 2006; Lin et al., 2007). One drawback of using ion-pairing reagent is suppression of ionization and compromise of sensitivity in LC–MS analysis. To alleviate this problem, post-column infusion of propionic acid and isopropanol or direct addition of acetic acid (0.5%) or propionic acid (1%) to the TFA-containing mobile phase has been shown to partially alleviate ion suppression caused by TFA (Apffel et al., 1995; Shou and Naidong, 2005). An alternative approach for chromatographic retention

of very polar compounds is porous graphitic carbon (PGC), the stationary phase of which is composed of large and flat sheets of hexagonally arranged carbon atoms. The retention mechanism on PGC involves dispersive interaction between analyte and mobile phase/stationary phase and dipolar and ionic interaction of the polar analyte with the polarizable graphite (Knox et al., 1986). PGC uses the same type of mobile phase as RPLC such as water, methanol, and acetonitrile, but it requires higher organic contents for elution, resulting in better retention for polar compounds (Hsieh et al., 2007).

Hydrophilic interaction chromatography (HILIC) has been gaining interest and utilization for analysis of polar molecules such as amino acids, neurotransmitters, peptides, and so on, since its name was first coined by Alpert in 1990 (Alpert, 1990) and practically advocated for LC-MS bioanalysis of polar analytes in the late 1990s in a review article in 2001 (Naidong et al., 2001). In HILIC, the analyte interacts with the stationary phase based on hydrophilic interaction and is eluted by a relatively hydrophobic binary mobile phase. The most commonly used stationary phases are bare silica, bonded phase such as cyano, diol, amino, and zwitterions. The mobile phase for HILIC typically employs water-miscible polar organic solvents such as acetonitrile or methanol, and water is usually the stronger eluting solvent. The gradient typically starts with high organic and low aqueous (5–10% aqueous) mobile phase and increases to 50–60% aqueous or even higher in extreme cases for elution of the analytes. HILIC is complementary to RPLC in that polar compounds are more highly retained than nonpolar compounds, and the elution order is usually the reverse of that on RPLC columns. It can provide good retention and unique selectivity for polar compounds. The high organic contents in the mobile phase when analytes are eluted can provide significantly increased sensitivity for polar analytes in comparison with RPLC.

Normal-phase liquid chromatography (NPLC) employs a polar stationary phase such as bare silica, amino-bonded or cyano-bonded silica, and a nonpolar, nonaqueous mobile phase such as hexane, methanol, and so on. The retention mechanism on NPLC involves adsorption of the analyte to the stationary surface by hydrophilic interactions (e.g., hydrogen bonding), and retention increases with the polarity of the analytes. The use of more hydrophobic solvent (e.g., hexane) will facilitate retention, while more polar solvent (e.g., methanol) are considered stronger mobile phase. NPLC is very challenging to operate because any variation of the trace levels of moisture in the mobile phase can significantly impact chromatography and must be strictly controlled. Nevertheless, NPLC can effectively retain and separate analytes that are readily soluble in nonpolar

solvents, such as fatty acids and steroids. NPLC can also more effectively resolve structural isomers because the interaction strength relies not only on the functional groups in the analyte molecule but also on steric hindrance factors. Therefore, an important field of application of NPLC for biomarker quantitation is chiral separation, which will be discussed in more detail in the following sections.

Ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and size exclusion chromatography (SEC) are important modes of separation for large molecules (Hong et al., 2012; Haverick et al., 2014; Fekete et al., 2015). IEC retains and separates ions or polar compounds based on their charge status. The stationary phase is typically a resin or gel matrix consisting of agarose or cellulose beads with covalently bonded charged functional groups. The analytes (anions or cations) are retained on the stationary phase by adsorption to functional groups with opposite charge and can be eluted by increasing the concentration of a similarly charged species in the mobile phase that will displace the analyte ions from the stationary phase. HIC separate biomolecules based on their reversible adsorption to the stationary phase according to their hydrophobicity. The samples are loaded at high salt concentration that reduces the solvation of the analytes and promotes their binding to the stationary phase. A decreasing salt gradient elution is then used to elute the analytes from the stationary phase in order of increasing hydrophobicity. While similar to what can be done by RPLC, HIC has the advantage of performing separation under non-denaturing conditions (i.e., physiological pH, without organic in mobile phase, and ambient temperature), where a collected fraction can be assessed for their biological function. SEC is a chromatographic method in which molecules in solution are separated as a result of their permeation into the matrix of a stationary phase such as agarose gel or polyacrylamide. Large molecules may be excluded from some or all of the porous matrices of the packing by their physical size and elute earlier than the smaller molecules, which have the opportunity to enter the pores. IEC, HIC, and SEC are rarely directly interfaced with mass spectrometers because the mobile phase used to elute analytes normally contains high concentrations of non-volatile buffers/salts and is not compatible with the ion source. IEC is often adopted as the first dimension of separation to couple with RPLC in two-dimensional liquid chromatography (2D-LC) systems for comprehensive analysis of complex components. HIC is mostly commonly used for separation and purification of therapeutic proteins such as monoclonal antibody for characterization purpose. SEC is often used for the analysis

and purification of synthetic and natural polymers, such as proteins, polysaccharides, or nucleotides.

Chiral separation plays an important role in the bioanalysis of biomarkers due to the isomeric nature of many types of endogenous molecules and their analogs. Resolution on chiral columns is achieved by different interaction behaviors of the two enantiomeric analytes with the single enantiomer (chiral selector) immobilized on the chiral stationary phase (CSP). The forces involved in the interaction are multifaceted, including polar/ionic interactions,  $\pi-\pi$  interactions, hydrophobic effects, and hydrogen bonding. These forces are very weak and are subject to many variables such as additives, pH, temperature, and composition of the mobile phases, all of which require careful optimization in order to achieve maximal selectivity. The chiral selector used as CSP for liquid chromatography (LC) can be classified into two types (Okamoto and Ikai, 2008). The first type consists of optically active small molecules, such as macrocyclic antibiotics, cyclodextrins, and crown ether, immobilized on silica gel or organic polymer gel. The second type consists of optically active polymers coated on silica gel, which are further categorized into synthetic polymers (such as polyamide) and natural polymers (such as polysaccharide and protein). Polysaccharide-based derivatives are currently the most popular chiral selectors due to their versatility, durability, and loading capacity. There are many examples of enantioseparations using this type of column in the literature (e.g., CHIRALPAK column) (Morin, 2009). For example, chiral separation of different enantiomers of lipid oxidation biomarkers demonstrated in Figure 4.1 was achieved by utilizing a CHIRALPAK AD-H column, for which the stationary phase is silica gel coated with amylose tris(3,5-dimethylphenylcarbamate). Chiral separations using polysaccharide columns are typically performed using NPLC because interactions involved in enantiomeric resolution on these CSPs is stronger under normal-phase conditions. One of the challenges of interfacing NPLC with MS is the possible explosion hazard when a high flow of flammable solvent, such as hexane, is introduced into the heated ionization source. Different approaches have been explored to overcome this issue, such as by using inert gases for nebulizing and desolvation processes, by reducing the ion source temperature, or by incorporating a large aqueous post-column makeup flow to reduce the hexane concentration in the mobile phase prior to the ion source. RPLC is simpler to operate than NPLC and has increasingly become a favorable choice for chiral separation. Most of macrocyclic CSPs work well under reversed-phase conditions. In addition, polysaccharide-based and protein-based reversed-phase chiral columns are also commercially available (Chen et al., 2005).

#### 4.2.4 Modern LC Technologies

##### 4.2.4.1 HPLC and UHPLC

The ever-growing number of sample numbers in the fast pace research environment presents the demand of improving analytical throughput. For quantitative high performance liquid chromatography (HPLC)–tandem MS (MS/MS) bioanalytical methods, short, narrow-bore (20–100 mm  $\times$  ~2.0 mm) columns with small particle sizes (3–5  $\mu\text{m}$ ), relatively high flow rates (e.g., >0.5 mL/min), and fast gradient elution have been routinely used to achieve analytical run times in single-digit minute time. Meanwhile, ultrahigh pressure LC (UHPLC) has been widely used to achieve higher throughput by increasing the speed of analysis while retaining or improving separation efficiency (Mazzeo et al., 2005). UHPLC is conducted by using sub-2  $\mu\text{m}$  particle analytical columns and specially designed instruments. Small particles have shorter diffusion path lengths, allowing analyte molecules to travel in and out of the particle faster with less resistance and spend less time inside the particle where peak diffusion can occur. Therefore, small particle columns provide increased separation efficiency and the ability to work at increased linear velocity without a loss of efficiency, leading to both better resolution and higher speed. In addition, reduced peak width and increased peak concentration can provide more sensitive MS detection. However, the benefit of utilizing small particle materials is accompanied by increased column backpressure, which is inversely proportional to the square of particle size. In the past decade, UHPLC systems have been developed by instrument companies to cope with the high backpressure resulting from small particle columns. Sub-2  $\mu\text{m}$  particle columns with diverse chemistry have also become increasingly available from almost all major column manufacturers. In bioanalytical applications, it has been demonstrated that UHPLC could achieve separations 2–20 times faster than HPLC while maintaining or improving resolution and sensitivity. Typically, UHPLC analysis of a single compound in a biological matrix using an internal standard requires 1–2 min/injection. The optimal mobile phase flow rate for UHPLC–MS experiments on 2.1 mm diameter columns should be between 0.3 and 0.6 mL/min, and higher flow rate could cause loss in sensitivity and slightly reduced separation efficiency.

Alternative to sub-2  $\mu\text{m}$  particles, partially porous or fused-core materials can be packed for columns for ultrafast separations. The fused-core particle consists of a thin layer of porous shell fused to a solid particle core. Compared with totally porous particles, the fused-core particles have a much shorter diffusion path for the analyte into and out of the stationary phase, resulting in

minimized peak broadening at high flow rate. Due to their larger particle size, the backpressure generated from a fused-core column is usually approximately half of that from sub-2 µm column with the same dimensions and operating conditions (Cunliffe and Maloney, 2007). The above characteristics allow reduction in analysis time without sacrificing resolution or the need to change the LC system to an ultrahigh pressure system.

#### 4.2.4.2 Miniaturized Column LC

Over the past years, there is increased trend of miniaturizing the columns for LC-MS-based bioanalysis. The typical column diameters routinely used have been decreased from 4.6 to 2 mm, and even 1 mm, and there is an increased interest in downsizing further to below 1 mm to 50 µm. The downsizing scale factor, defined as the squared ratio of the column inner diameters, can be theoretically applied to all system parameters, including the flow rate, injection and detection volumes, and connecting tubing diameters (Chervet et al., 1996). One of the major motivations for reducing the column size is for enhanced ionization efficiency. It was discovered that in the electrospray process, the lower flow rate reduces the size of the charged droplets, which therefore results in improved evaporation and fission cascade and higher ionization efficiency and less ion suppression (Wilm and Mann, 1994). An important finding was that the ion suppression is practically absent at flow rates below 20 nL/min (Schmidt et al., 2003). Other advantages of using miniaturized LC are reduced mobile phase consumption, as well as less sample consumption, particularly in the case of limited sample volumes. HPLC miniaturization present serious challenges for designing and manufacturing of the instrumentation: the pumps must be able to deliver low flow rate (nL/min to µL/min) with minimized pulsation and accurate gradient. Tubing, unions, valves, column, sample introduction system, and detector must present compatible volume to prevent band broadening. Currently, two main types of pump devices are commercially available: syringe pumps and dual piston reciprocating pumps (Nazario et al., 2015). The former ones are able to directly deliver expected flow without splitting. For reciprocating pumps, there are splitless designs such as those from Sciex, Thermo, and Waters, and split design such as Agilent 1200 Series system, which consume more solvent than splitless system.

As many LC columns with different diameters and lengths are being developed over the years, general classification of columns has been proposed (Table 4.1) (Saito et al., 2004). The option of stationary phases for nano-, capillary, and micro-LC is not as many as conventional HPLC. Nevertheless, the most popular ones such as C18, C8, C4, phenyl, HILIC, pentafluorophenyl (PFP),

**Table 4.1** General classification of LC columns.

ID column (mm)	Classification	Typical flow rate
3.0–5.0	HPLC	1–2 mL/min
2.1	Narrow-bore HPLC	0.5 mL/min
0.5–1	Micro-LC	0.1 mL/min
0.1–0.5	Capillary LC	1–20 µL/min
<0.1	Nano-LC	<1 µL/min

Source: Saito et al. (2004). Reproduced with permission of John Wiley & Sons.

and so on are available from different vendors. The most used particle sizes are 3–5 µm and length ranges from 50 to 500 mm. In the last decades, microfluidic technology has become very popular, and several chip-based columns are commercially available, such as those from Agilent, Waters, Sciex, and New Objective (Yin et al., 2005). The chips from Agilent and Waters have the integrated enrichment column, analytical column, connecting lines, and nanospray emitter onto a single chip device. All the commercial chip-based columns require specialized interface. On the other hand, the “plug-and-play” format has the advantages over conventional miniaturized column of ease of operation, elimination of fitting, unions and other dispersive element, and therefore improved robustness and reproducibility.

In recent years, nanoflow UHPLC (nUHPLC) that employs particles with sub-2 µm particles or sub-3 µm superficially porous particles has been applied but mainly in proteomics research (Sestak et al., 2015). All principles of an accelerated separation performed on analytical column packed by smaller particles and at ultrahigh pressure are applicable to miniaturized columns. nUHPLC conditions have been shown to be a practical way of speeding up the time-consuming nano-LC analysis of complex samples in proteomics and metabolomics research (Nováková et al., 2013; Jones et al., 2014).

The true advantages of using miniaturized column for targeted biomarker bioanalysis remains to be proved (Hilhorst et al., 2014). Nano-, capillary, and micro-LC still remains not popular in routine bioanalytical applications due to many reasons: lengthy run time, difficulty in locating and stopping leakage, ease of clogging, lack of robustness, unstable spray on MS leading to poor reproducibility, and higher technical skills required for the operating analyst. More importantly, the sensitivity gain on a miniaturized column is often hampered by limited injection volume and loading capacity, which reduced the amount of sample that can be analyzed. Often, the loadability is decreased to the extent that no net sensitivity gain can be obtained by reducing the column diameters.

It is often argued that this limitation can be overcome by applying pre-concentration techniques such as pre-column trapping. However, if the properties of the analyte allows such pre-column focusing, the same strategy can be applied for a conventional size LC system without the challenges associated with miniaturized columns, given that enough sample is available, which is usually the case for targeted biomarker quantitation. Therefore, sensitivity gain that can be achieved by downsizing column diameter is more valuable in the situations with limited sample volume, such as analysis of samples from small animals, infants, or special matrices, which may be the situation for research of some biomarkers. In general, miniaturized columns have been much more widely used for omics types of biomarker discovery, where there is often the requirement for profiling of complex samples with superior sensitivity from limited sample volume. In contrast, for routine targeted quantitation of known biomarkers, regular flow HPLC and UHPLC are the instruments of choice due to their robustness, reducibility, ease of operation, and high throughput capability. Due to above reasons, there are limited examples of utilization of miniaturized column for targeted biomarker quantitation in biological matrices. In one example of quantifying human osteopontin as a cancer biomarker, a microflow LC–MS/MS platform was used (Faria et al., 2015). The method involved immunocapture of the analyte protein using antibodies followed by trypsin digestion to obtain the signature peptide. Analysis was carried out on a nUHPLC system coupled with chip-based Waters ionKey separation device (100 mm × 150 µm I.D., 1.7 µm) using a flow rate of 2.5 µL/min. By using a trap column (50 mm × 300 µm), 20 µL of samples can be injected. The micro-LC system allowed use of smaller sample volume, dilution of samples to avoid matrix interference, and reduced solvent consumption. In another example of analyzing neuropeptides in brain microdialysates, an nUHPLC system was employed (Maes et al., 2015). The analysis was carried out on a BEH130 C18 column (100 mm × 100 µm I.D., 1.7 µm) using a flow rate of 300 nL/min. The samples were concentrated on a C18 Trap column (10 mm × 180 µm I.D., 5 µm) with injection volume of 5 µL. Sensitivity of 0.5 and 3.0 pM was achieved for neuropeptides (MW 1672.9) and neuromedin B (MW 1132.3), respectively.

#### 4.2.4.3 2D-LC

Two-dimensional LC (2D-LC) by coupling different separation mechanisms is often used for the analysis of multicomponent mixtures of extreme complexity. Theoretically, peak capacity of a two-dimensional separation is equal to the product of the peak capacities of the individual dimensions if they are 100% independent to each other (orthogonal). Therefore, separation

efficiency can be drastically improved by combining two modes of separations that are based on orthogonal mechanisms. 2D-LC could be operated in either offline or online configuration. In offline 2D-LC mode, the effluent from the first dimension column is collected manually or via a fraction collector and reinjected onto the secondary column. Either a part or the entire components from the first dimension can be analyzed on the second dimension. Online 2D-LC can be classified into heart-cutting or comprehensive LC. In heart-cutting 2D-LC, only the relevant part of the effluent, containing the target analytes, is directed to the second dimension via an online interface. In comprehensive 2D-LC, the entire effluent from the first dimension is transferred to the second dimension for analysis, which required complicated valve switching configurations and complex instrument and software settings. Due to its ability to separate complex components, 2D-LC is particularly valuable in untargeted profiling of large number of analytes for identification of novel biomarkers, such as metabolomics, lipidomics, or proteomics research (Nie et al., 2010). For targeted bioanalysis, it should be noted that 2D-LC is rarely necessary unless extreme sensitivity is needed or in the case when co-eluting components cannot be separated on single dimensional chromatography.

## 4.3 Mass Spectrometry

### 4.3.1 Major Types of MS Used for Targeted Biomarker Quantitation

In the past decades, there has been significant improvement in the MS in multiple aspects: sensitivity, dynamic linear range, mass range, mass resolution, acquisition speed, and the instrument cost (Himmelsbach, 2012). Triple quadrupole MS remains the instrument of choice for routine and high-throughput targeted analysis of biomarker in biological samples due to its outstanding performance when quantitation is needed. Meanwhile, development in high-resolution MS and hybrid MS has enabled more novel and versatile data acquisition that provides advantages of better specificity and acquisition of additional information beyond targeted quantitation.

In quadrupole mass spectrometers, the Q1 mass analyzer filters ions with the desired mass-to-charge ratio ( $m/z$ ) so that these ions are fragmented within Q2 via collision-induced dissociation (CID) and their product ions are resolved by Q3 before reaching the detector. By specially monitoring selected product ions generated via CID from targeted molecular ions, a process called selected reaction monitoring (SRM) or MRM, triple quadrupole instruments provide superior selectivity and sensitivity against background noise originating from

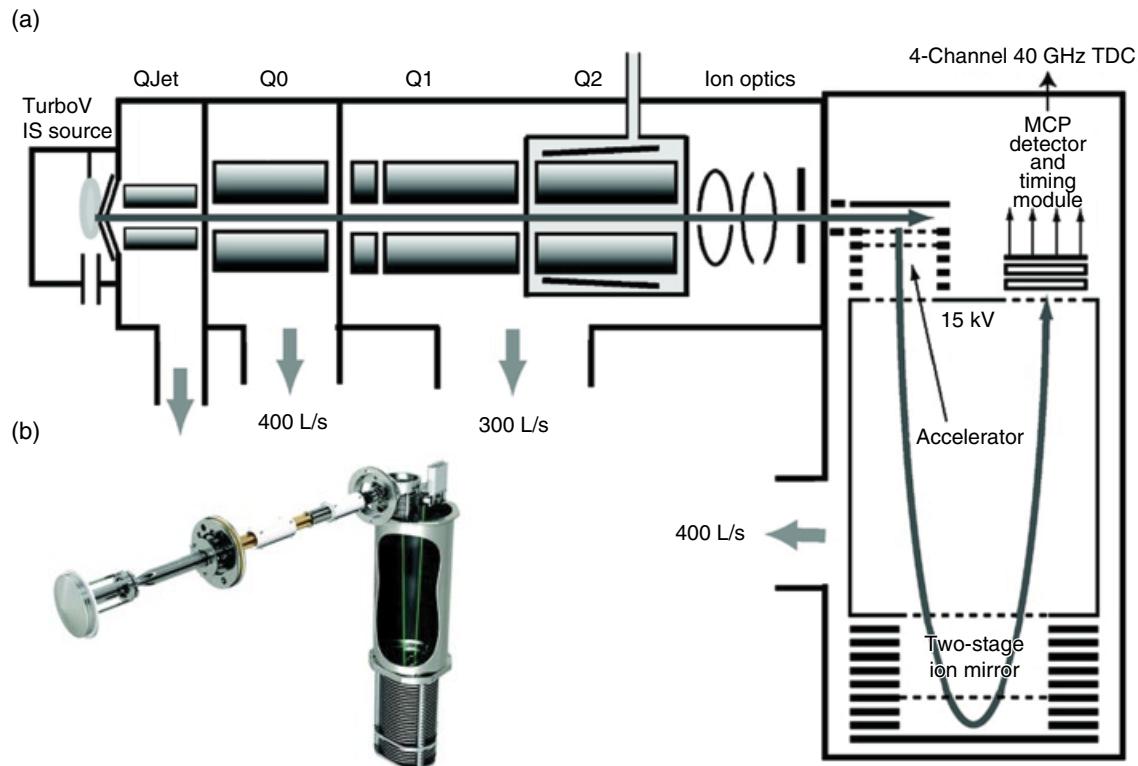
chemical and biological matrices. Modern triple quadrupole instruments can rapidly switch between multiple SRM transitions within a single acquisition cycle. In addition, the method can be scheduled to monitor the MRM only around the expected retention time of each analyte, therefore increasing the total number of MRM transitions during a single run. As a result, a large number of analytes can be measured from a single injection, which is particularly valuable for investigating multiple biomarkers related to a certain biological pathway, for example, subsets of hormones or bile acids (Han et al., 2015; Sinreich et al., 2015). For analytes that are resistant to fragment in CID, an alternative data acquisition mode on triple quadrupole instrument is pseudo-MRM, in which same ion (the molecule ion) is monitored in Q1 and Q3. The intact molecule ion can be preserved, while the interference components are fragmented in Q2, allowing selective detection of the analyte with reduced noise (Ciccimaro et al., 2014).

Ion trap (3D trap) and linear ion trap (2D trap) mass spectrometers, featuring full MS scanning, data-dependent MS/MS acquisition, and MS<sup>n</sup> spectral acquisition, were used to be the cornerstone instruments for qualitative analysis. Ion trap instruments have higher scan speed and more sensitive full MS scanning than triple quadrupole instruments because of their ability to accumulate ions within the trap without ion filtering. Ion trap instrument as stand-alone instrument is less relevant for targeted biomarker quantitation. There are limited examples of utilizing ion trap type of instrument for targeted quantitation of biomarker. A work of quantitation of creatinine and cortisol in serum using LTQ linear ion trap instrument showed high precision and good reproducibility of the system, qualifying it as an instrument for measurement of analytes in complex matrices (Dai et al., 2008). Nevertheless, due to recent advance of high-resolution mass spectrometers with respect to technology, operation, and price, they are promising to replace ion trap or linear ion trap instruments as a major MS platform for qualitative analysis.

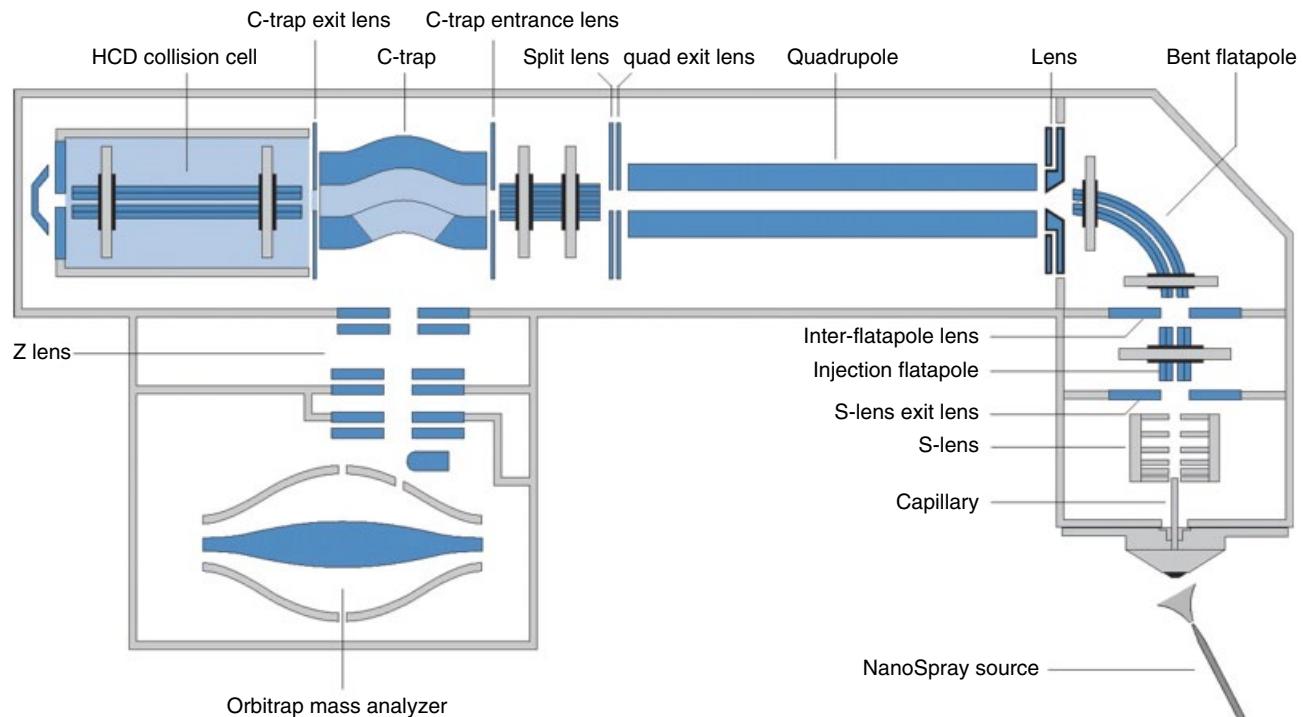
Hybrid triple quadrupole-linear ion trap (Q-Trap) features ion path of a standard triple quadrupole MS, but the final quadrupole can be operated either as a conventional quadrupole mass filter or as linear ion trap. Q-Trap instrument provide scan functions identical to those of classical triple quadrupole and those similar to an ion trap mass spectrometer (Hopfgartner et al., 2004; King and Fernandez-Metzler, 2006). This unique function allows the Q-Trap to be operated either as a stand-alone triple quadrupole instrument or as an ion trap MS. More importantly, MRM scan on Q-Trap instruments can serve as survey scans to trigger the information-dependent acquisition (IDA) of enhanced product ion (EPI) spectra, which provide product ion mass spectra

with rich fragmentation information for structural identification. The Q-Trap mass spectrometer has been considered a unique and versatile LC-MS platform that is well suited for both quantitative analysis and structural identification studies. The method can be set up on Q-Trap for simultaneous detection and confirmation of large number of compounds in biological fluid in a single analytical run by a scheduled MRM scan, which triggers IDA of EPI. The identification of the compounds can be confirmed by comparing MS/MS spectra against library containing the spectra of known compounds.

High-resolution mass spectrometry (HRMS), which can provide resolution greater than 10,000 (mass of the peak/full width at half maximum height) and accurate mass measurement (within 10 ppm), has emerged as the novel MS platform for biomarker analysis. The exact mass measurement can provide highly specific analysis, and the full-scan HRMS function has been routinely used for profiling or screening purpose. In addition to small molecule analysis, HRMS is particularly valuable for analyzing large molecules such as peptides, proteins, and oligonucleotides at intact level (Morin et al., 2013; Wang and Ji, 2016). There are two main types of commercial high-resolution mass spectrometers that are commonly used in modern laboratories for biomarker analysis: time-of-flight (TOF) and Orbitrap, both of which are available in hybrid formats. Q-TOFs combine the Q1 mass filter and Q2 collision cell from triple quadrupole mass spectrometers with a TOF region as the second mass analyzer (Figure 4.2) (Andrews et al., 2011). Orbitrap operates by radially trapping ions around a central spindle electrode and recording the oscillations of the ions, which are transformed into mass spectra using Fourier transformation. In commercial platforms, Orbitrap is either combined with an external accumulation region such as a linear ion trap (LTQ-Orbitrap) or Q1 mass filter (Q-Exactive) (Figure 4.3) (Michalski et al., 2011). The major difference between TOF and Orbitrap is the impact of resolving power on the duty cycle. For TOF MS, the resolving power (usually between 20,000 and 40,000) is independent of the duty cycle, while for an Orbitrap instrument, increasing resolving power requires longer time for trapping/recording the oscillations of the ions and it can provide resolution up to 240,000 (Hopfgartner, 2011). Hybrid TOF instruments have been increasingly used for quantitation in recent years (Zhang et al., 2009; Dillen et al., 2012). Full-scan HRMS provides accurate mass spectra of the analyte ions, which can be processed to generate extracted ion chromatogram (EIC) for quantitation. Alternatively, MRM<sup>HR</sup> mode, also named as parallel reaction monitoring (PRM), can be used to acquire HRMS spectra of product ions from certain precursor ions that were selected in Q1, and specific product ions can be processed to generate EIC for quantitation with improved



**Figure 4.2** Construction details of the TripleTOF MS. (a) A detailed illustration of the major platform features. (b) An image of the machined TripleTOF MS instrument platform. Source: Andrews et al. (2011). Reproduced with permission of American Chemical Society.



**Figure 4.3** Construction details of the Q-Exactive MS. This instrument is based on the Exactive platform but incorporates an S-lens, a mass selective quadrupole, and an HCD collision cell directly interfaced to the C-trap. Note that the drawing is not to scale. Source: Michalski et al. (2011), <http://www.mcponline.org/content/10/9/M111.011015.short>. Used under CC BY 4.0 <https://creativecommons.org/licenses/by/4.0/>.

selectivity and sensitivity (Peterson et al., 2012; Sturm et al., 2016; Zhou et al., 2016).

There are significant advantages of using HRMS for quantitation purpose (Ruan et al., 2011). The full-scan HRMS can be performed without prior knowledge of the MRM transition of the analyte, therefore avoiding specific tuning and method setup. More importantly, non-targeted data acquisition under full MS scan allowed the opportunities to conduct post-acquisition data mining to reveal other components in the injected samples, such as biologically relevant molecules and analogs, which may be of particular interest to biomarker research. In one example of using full-scan HRMS to relatively quantify different glyco-isoforms of ApoC3, in addition to obtain the quantitative information of the targeted analytes, the data can be processed to reveal existence of ApoC1 and its truncated form (Jian et al., 2013). In the field of biomarker analysis, even though HRMS has been at this moment mainly used for omics type of profiling research for biomarker discovery, it has shown great promises as a versatile platform suitable for both screening and targeted quantitation in a single experiment (Gertsman et al., 2014).

#### 4.3.2 Ionization Techniques

Coupling LC effluent with MS requires ionization under atmospheric pressure. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the liquid introduction sources that have the most applications in the field of bioanalysis. In ESI, charged droplets are formed by spraying the sample solution through a high voltage capillary in the presence of a strong electric field. The charged droplets move toward the mass spectrometer inlet, generating analyte ions during evaporation and droplet fission (Enke, 1997). In ESI, the ionization occurred in liquid phase. It has been shown to be suitable for ionic and polar analytes, with a very high success rate for most biomarker molecules, including small and large molecules. In contrast, ionization for APCI occurs in gas phase. In APCI, a heated pneumatic probe is used for nebulization, and a high voltage needle is used to produce a corona discharge for ionization of the evaporated solvent and for reaction with the analytes. APCI allows ionization for neutral compounds with relatively smaller size (MW < 1000). It is a more harsh ionization technique and may cause the compounds to break down in ion source. It is often used for enhanced ionization of neutral compounds, for larger dynamic range, and to overcome matrix effects (Choo et al., 2005). In recent years, dual ion source consisting distinct or integrated probes for both ESI and APCI has become commercially available, the latter of which enabled analysis of compounds of large range of ionization properties under both modes simultaneously.

Atmospheric pressure photoionization (APPI) is a soft ionization technique that was developed for those molecules poorly amenable to ESI and APCI (Marchi et al., 2009). APPI is based on interaction of a photon beam generated by a UV discharge lamp with the gas-phase vapors formed by the nebulization of the LC effluent. The absorption of a photon by a molecule may lead to an electronically excited molecule that releases an energetic electron and generates a positively charged ion. In some cases, a preferentially ionized substance called a dopant has to be added in large amount either to the mobile phase or by post-column addition to increase ionization efficiency. Dopant acts as an intermediate between photons and the analyte by ways of charge exchange or proton transfer. Toluene and acetone are the most frequently used dopants. APPI has gained popularity due to its capability to ionize both polar and nonpolar compounds. In one example of quantifying cortisol and cortisone in plasma and serum, the APPI ion source with toluene used as a dopant was shown to improve the signal to noise by a factor of three compared to APCI (Kushnir et al., 2004). In another example of biomarker quantitation, APPI was used to improve the sensitivity of 27-hydroxycholesterol in small volume of plasma. Unlike the conventional LC-ESI-MS or the standard GC-MS workflow, this method eliminated the need for derivatization (Karuna et al., 2009).

#### 4.3.3 Ion Mobility

There has been considerably increased interest in interfacing ion mobility MS (IMS) with front end of MS for improved analytical power. IMS separates ions based on their size-to-charge ratios, which represents an analytical tool for investigating molecule structures when added to MS. More importantly, from bioanalytical point of view, IMS provides separation of isomers, isobars, and conformers and can reduce chemical noises and other interferences. There are three main types of IMS devices (Himmelsbach, 2012): (i) drift-time ion mobility spectrometry (DTIMS); (ii) differential mobility spectrometry (DMS), marketed by Sciex as SelexION and by Thermo Fisher Scientific as field asymmetric waveform ion mobility spectrometry (FAIMS); and (iii) traveling-wave ion mobility spectrometry (TWIMS) as a trademark by Waters. In DTIMS, ions are moving through a homogeneous and continuous electric field in a drift tube in the presence of a neutral gas. The time it takes for the ions to move along the tube is directly proportional to the ion's collision cross section. DTIMS provides highest resolving power but lacks sensitivity due to its low duty cycle. It is mainly used in combination of direct infusion to determine cross sections of large molecules. DMS is a method to separate ions based on the difference between ion

mobility in high and low electric fields in gases at or near atmospheric pressure. Ions are continuously introduced into an ion mobility spectrometer and travel between two electrodes. Separation voltages are applied across the ion transport channel, causing the ions to migrate toward the walls and leave the flight path. Their trajectory is corrected by a compensation voltage to focus certain ions and transport them into MS. In DMS, the ions are monitored in a continuous fashion, which makes it best suitable to act as ion mobility filter for targeted analysis of specific analytes. Modifiers, typically isopropanol, acetonitrile, or methanol, are often added to the gas, transporting the ions. As the ions entering the IM devices form clusters with the modifier, their mobility characteristics are changed and the separation power may be enhanced. TWIMS uses a sequence of symmetric potential waves continually propagating through a drift tube to drives ions based on their mobility. Smaller ions collide less with gas and move faster, while larger ions collide more and get delayed. TWIMS has low resolving power but demonstrated good sensitivity. In theory, each of the IMS can be interfaced with a variety of MS. TOF analyzers are used most frequently, but there is increased combination with quadrupole and ion trap/hybrid ion trap instruments. DMS devices, commercially integrated into MS systems, have often be used in combination with LC to improve the performance of bioanalytical methods. In one example of simultaneous determination of prostanoids and thromboxane B<sub>2</sub>, FAIMS reduced background noise, separated isobaric PGE<sub>2</sub> and PGD<sub>2</sub>, and the dynamically interchanging thromboxane B2 anomers, two diastereomers formed by opening and close of lactol ring in the structure, without changes to sample preparation or chromatography (Kapron et al., 2006). It should be noted that matrix effects, the suppression or enhancement of ionization caused by other components in the samples, cannot be altered by ion mobility device. It is generally believed that matrix effects happen during ionization in the source region, and this is before the ions enter the ion mobility device. In addition, the absolute signal intensity acquired on the instrument with an ion mobility device in place is usually lower than without the device, due to the added traveling path of the ions, although signal to noise may be improved because of enhanced selectivity.

#### 4.3.4 Fragmentation Mode

In MS/MS analysis, the efficient fragmentation of precursor ions into characteristic product ions is of particular importance for specific detection of targeted analytes, as well as for structural elucidation and confirmation. The most commonly used method of achieving fragmentation is CID. In CID, the precursor ions are accelerated with electrical potential to high kinetic

energy and allowed to collide with ground-state neutral gas, causing the kinetic energy to convert into internal energy, which in turn results in cleavage of bonds in the ions. Higher energy collisional dissociation (HCD) is a type of CID specific for the Orbitrap system (Olsen et al., 2007). In HCD, the ions are fragmented in collision cells rather than the ion trap and then transferred back through the C-trap for analysis at high resolution in Orbitrap (Figure 4.3). In comparison with CID in traditional ion trap instrument, HCD features no low-mass cutoff, high resolution detection, and increased fragmentation efficiency, resulting in better quality MS<sup>2</sup> spectra. CID is a fast dissociation method and amenable for both ion trap types (activation time  $\geq 10^{-3}$  seconds in ion trap, Orbitrap) and “beam-type” (activation time  $10^{-5}$  to  $10^{-4}$  in triple quadrupole and Q-TOF) instruments and therefore has been the default method of performing ion dissociation for MS/MS detection, including quantitative bioanalysis of biomarkers.

Different from CID, which generate ion excitation that is predominantly vibrational in nature, electron capture dissociation (ECD) induces dissociation through the electronic excitation of the ions (Hart-Smith, 2014). In ECD, multiple charged positive ions are irradiated with low energy electron to produce radical cations, which dissociate to generate fragments. The efficiency of the ECD process requires that the precursor ions be immersed in a dense population of near-thermal electrons and can be only performed in the Fourier transform ion cyclotron resonance (FTICR) MS. Thermal ions introduced into the radio-frequency electrostatic field of ion trap and quadrupole instrument maintain their thermal energy only for a fraction of a microsecond and are not trapped. For this reason, electron transfer dissociation (ETD) has been invented. In this approach, negatively charged radical reagent ions are used to induce electron transfer, and the dissociation pathways follow the same process as those in ECD. Currently, the polycyclic aromatic hydrocarbon compound fluoranthene (C<sub>16</sub>H<sub>10</sub>) is the preferred reagent molecule. ETD is available on a relatively wide variety of instrument platforms, including stand-alone ion trap, LTQ-Orbitrap, Q-TOF, and Q-FTICR. ETD is readily achieved in the order of milliseconds using commercially available instruments and showed great utility in LC-MS/MS analysis of complex samples. ECD/ETD as alternative fragmentation approach has showed large impact for the field of proteomics research. In ECD/ETD, dissociation of radical cations occurs without energy distribution, which means that the cleavage does not necessarily favor a molecule's weaker bonds. For peptides and proteins, this induces backbone cleavage without loss of posttranslational modification, such as phosphorylation, glycosylation, sulfonation, and nitrosylation, which is particularly

important for structural characterization (Frese et al., 2011; Kim and Pandey, 2012). However, the application of ECD/ETD in small molecule analysis and targeted biomarker quantitation is very limited. Unlike CID, only ions of charge states  $\geq 2$  can be analyzed by ECD/ETD because capture of an electron by a singly charged precursor ion results in an uncharged species.

#### 4.3.5 Emerging MS Techniques

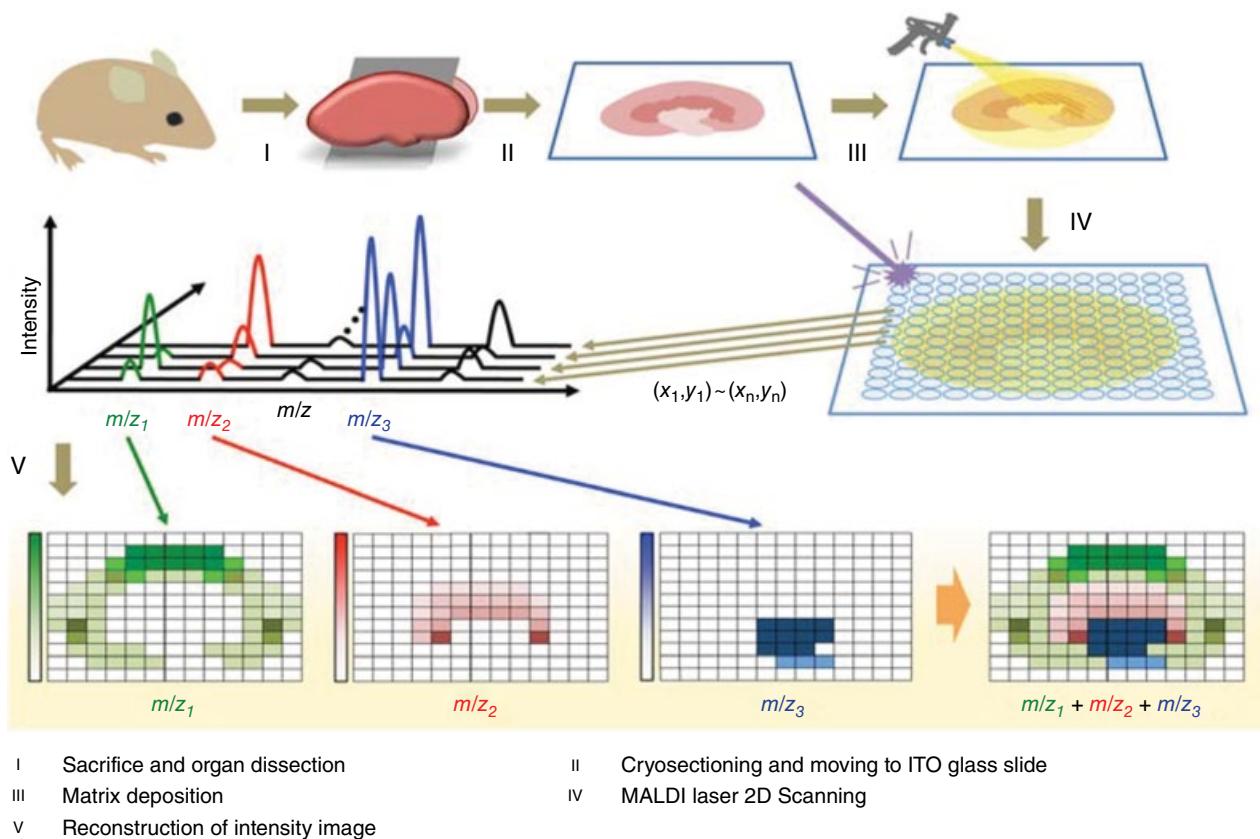
##### 4.3.5.1 MS Imaging

MS imaging (MSI) allows direct analysis of the spatial distribution of various molecules in tissue without the needs for radioactive or fluorescent labeling normally used in histochemical techniques. It has emerged as one of the powerful techniques to visualize endogenous metabolites, lipids, peptides, and proteins, as well as drugs and their metabolites *in situ* (Aichler and Walch, 2015; Sugihara et al., 2016). Currently, the three main types of ionization techniques used for IMS are matrix-assisted laser desorption/ionization (MALDI), secondary ion mass spectrometry (SIMS), and desorption electrospray ionization (DESI). In the workflow of MALDI, the surface of interest, a tissue section, is coated with a

matrix for extracting molecules from the tissue into the matrix. Radiation of the matrix layer with a laser desorbs the analyte molecules for MS analysis. The typical MALDI workflow consists of the following:

- i) Tissue preparation: Harvesting and snap freezing the tissue samples, which are then sectioned using a cryotome.
- ii) Matrix application: Coating the tissue with the matrix.
- iii) Data acquisition: The tissue samples are raster-scanned to generate mass spectrum for each spot.
- iv) Data analysis and image construction: Ion intensity map is generated from the data (Figure 4.4) (Fujimura and Miura, 2014).

The most critical step is the matrix application, which can significantly affect the type of molecules that can be detected, the ionization efficiency, and the detection reproducibility. MALDI was initially developed to image large biomolecules such as peptides and proteins, because of the interference of matrix ion in low  $m/z$  region of the mass spectrum. In recent years, MALDI imaging analysis of a wide variety of low molecular weight molecules such as lipids, endogenous metabolites,



**Figure 4.4** The schematic representation of MALDI-MSI experimental procedures. ITO, indium tin oxide. Source: Fujimura and Miura (2014), <http://www.mdpi.com/2218-1989/4/2/319>. Used under CC BY 3.0 <https://creativecommons.org/licenses/by-nc-sa/3.0/>.

and drugs have gradually increased due to combination with triple quadrupole, linear ion trap, or high-resolution MS detection for improved specificity (Norris and Caprioli, 2013; Trim and Snel, 2016). In SIMS, a sample surface is bombarded with an energetic primary ion beam. As the ion beam strikes the surface, a collisional cascade leads to ejection of materials from the surface, which contains ionized species. Same as MALDI, SIMS is a vacuum technique that requires sophisticated instrumentation and also confined the format of the samples that can be placed in front end of the MS. Unlike MALDI, SIMS does not require application of matrix, although it has been shown that use of MALDI matrix can improve sensitivity. SIMS is mostly successful for targeted analysis of inorganic compounds or biomolecules with relatively lower molecular weights, such as lipids, though extensive fragmentation associated with this ionization technique limits its ability to acquire comprehensive lipid profile (Ellis et al., 2013). DESI is an electrospray-based ionization technique that is conducted under atmospheric condition (Takats et al., 2004). In DESI, a spray of charged droplets is directed toward the sample surface. When the spray impacts the sample, a thin layer of solvent is formed, which dissolves the analytes. As the primary droplets arrive at the sample surface, they generate secondary microdroplets containing the dissolved analytes that are sucked into the MS. The main advantage of DESI is its ability to directly analyze surface under ambient conditions with minimal sample preparation. It also allows the soft ionization with minimal fragmentation of biomolecules such as lipids and proteins (Wu et al., 2010b). It is ideal for imaging in the low mass region, without interference from added matrix (as in MALDI) or fragmentation of large molecules (as in SIMS). It has been demonstrated that small neurotransmitters such as epinephrine and norepinephrine, antioxidants like ascorbic acid, and different types of lipids in adrenal gland can be successfully imaged by DESI (Wu et al., 2010a).

Each of these above techniques presents unique aspects in terms of vacuum requirement, sample preparation requirement, spatial resolution, analysis speed, analyte type, sensitivity, and reproducibility, many of which depend on detailed methods and parameters applied in each specific case. Simplistically, spatial resolution is often used as a single defining characteristic of performance of MSI. Spatial resolution is mostly limited by the focalization of the energy source, that is, the primary ion beam for SIMS and DESI and laser for MALDI. In addition, there is always a trade-off between the time required for data acquisition and the achievable resolution, with time increasing quadratically as the resolution is increased. In general, SIMS provides the best spatial resolution among the three techniques, routinely going

down to 2  $\mu\text{m}$ , and can be as low as 100 nm (Carado et al., 2008; Touboul and Brunelle, 2016). MALDI is typically performed at a resolution of ~25–100  $\mu\text{m}$  (Ellis et al., 2013), while there are studies showing analysis of drug compounds in tissue with spatial resolution down to 10  $\mu\text{m}$  (Rompp et al., 2011). For DESI, the achievable spatial resolution is limited by the diameter of the spray at the surface and is typically 200–500  $\mu\text{m}$ .

MSI has brought new insight into biomarker research by providing the capability to visualize distributions of proteins, lipids, cellular metabolites, drugs, and drug-related metabolites—therefore the potential to reveal physiological process, pathological mechanism, toxicological characteristics, and drug action. An important area is simultaneous localization of the drug and its targets in the tissue. For example, following administration of an enzyme inhibitor, the substrates or products of the targeted enzyme, or the downstream/upstream molecules in certain biological pathways of that enzyme can be analyzed at the same time with the inhibitor by MSI for more comprehensive understanding of the molecular changes that occur in tissue. One type of molecules of particular interest is lipids that are highly amenable to detection by MSI. In one example of MALDI imaging study of drugs that modulate lipids pathways, correlation of a small molecule sphingosine kinase 2 (SK2) inhibitor, the product of SK2, and the downstream pathway effector ceramides and glycosphingolipids was observed in a xerograph tumor model (Jones et al., 2015).

To meet the increased demand for biomarker research, MSI needs to continue improving the sample preparation, sensitivity, and quantitation aspects of the workflow. Particularly, accurate/absolute targeted quantification from tissue section requires additional research effort. Quantitative MSI on tissue has to deal with several fundamental aspects such as analyte recovery from the tissue and ionization matrix effects (Porta et al., 2015). The use of suitable calibration standard and internal standard therefore is critical. As with LC–MS-based quantitation techniques, calibration standard curves are required for quantitative MSI. There are two commonly used practices: spiking tissue homogenate with standards or spotting the standard series onto the control tissue section, the former of which addresses both analyte extraction and ionization efficiency, while the latter does not account for droplet dispersion on the tissue and does not reflect the actual analyte concentration per gram of tissue. Internal standards, particularly stable isotope-labeled internal standards, have been shown to effectively compensate for matrix suppression for quantitation of both endogenous and exogenous compounds by MSI (Pirman and Yost, 2011; Kallback et al., 2012). Combining with triple quadrupole

MS operated in MRM mode, MSI can provide high sensitivity and specific quantitation (Wagner et al., 2008; Lesur et al., 2012).

#### 4.3.5.2 Other Surface Analysis MS Techniques

In recent years, there has been an increased interest in application of dried blood spot (DBS) for drug discovery and development studies. Researchers have been exploring potentials of analyzing DBS directly from the paper by surface analysis techniques such as DESI, direct analysis in real time (DART), and paper spray, therefore eliminating the labor-intensive and time-consuming process of punching the paper and extracting the analyte (Wagner et al., 2014). In DART, the excited metastable helium gas molecule interacts with atmospheric components such as water and oxygen. This forms protonated water clusters (in positive mode) or  $O_2^-$  (in negative mode), which then transfer proton/charge to the analyte (Harris et al., 2011). DART does not require sample preparation and materials can be analyzed in their native state. Solid object can be presented in the front of ion source for direct analysis, and liquid can be dipped into a solid object, which is in turn analyzed directly. In one research of using DART for newborn phenylketonuria screening, the concentration of phenylalanine in DBS was determined using MRM mode with the internal standard *N,N*-dimethylphenylalanine (Wang et al., 2013). The results showed that this assay as a newborn screen for PKU can be performed in 18 seconds/sample with accuracy and precision and therefore can be effectively applied on a routine basis to analyze a large number of samples in PKU newborn screening and PKU patient monitoring. One of the major limitations of most ambient ionization methods is poor sensitivity toward DBS, in which the samples are distributed through the 3D porous structure of the blood card paper. Paper spray generates interest largely because it is able to achieve useful detection limits for the direct analysis of DBS (sub ng/mL in many cases) (Takyi-Williams et al., 2015b). In paper spray, the paper is cut to a sharp point either before or after spotting of the samples, with the dried matrix spot positioned 5–10 mm away from the tip. The sharp tip is positioned in front of the atmospheric inlet of the MS. An aliquot of solvent is applied to the paper to interact with the sample and to extract the soluble components. A high voltage (3–5 kV) applied to the paper initiates electrospray ionization from the tip of the paper. Paper spray requires no sample preparation and consumes only very small volume of samples (0.5–15  $\mu$ L). Multiple studies have demonstrated analysis of various small molecule analytes from blood, plasma, urine, and oral fluid with detection limit of single digit ng/mL or sub ng/mL (Takyi-Williams et al., 2015a; Manicke et al., 2016). While most of the applications were for quantitation of therapeutic drugs, one recent study successfully

extended the technique to monitor the activity of alanine transaminase (ALT) enzyme, a key biomarker for the detection of liver injury in patients. Blood samples from the patients were incubated with ALT substrates for 10 minutes, followed by spotting of the samples (4  $\mu$ L) on the paper. The concentration of pyruvate, the enzymatic product of ALT, was analyzed using paper spray-MRM analysis and correlated to ALT activity in the patients. This simple method eliminates complicated enzymatic activity assays and enable immediate monitoring of liver function after administration of a specific drug (Damon et al., 2016).

## 4.4 Summary and Future Perspectives

LC-MS has the capability to provide sensitive, selective, and fast bioanalysis and its power has been increasingly recognized by scientists in biomarker community. LC allows separation of complicated components in the biological samples including isobaric, isomeric, and interference peaks, which could be a prominent priority for biomarker analysis. There are more and more varieties of LC modes and column chemistry available for separation of biomarkers of different natures, such as very polar amino acids, hydrophobic lipids, high molecular weight proteins, and so on. To meet the ever-increasing demands of fast and efficient analysis, advanced LC techniques such as UHPLC and multiplex LC have been applied widely. Miniaturization of LC column has been the trend in recent years, and narrow-bore and micro-HPLC with fast gradient elution are now routinely used to achieve fast run time. For quantitation of trace level of endogenous molecules in samples of limited volume, nano-LC has demonstrated its advantages though it has not been widely utilized in bioanalytical laboratories. For targeted bioanalysis, triple quadrupole MS operated in MRM mode is the instrument of choice. Advancement in the instrumentation and software has made it possible to monitor hundreds of analytes in a single run on a triple quadrupole instrument. Alternatively, HRMS has recently gained significant popularity as a quantitation platform. Ease of method setup, high specificity, the capability to acquire quantitative/qualitative data simultaneously, and the potential of data mining have made HRMS such as TripleTOF MS and Q-Exactive favorable choice for biomarker quantitation, particularly when large panel of analytes are involved. Another important field of application of HRMS is large molecule quantitation, such as peptides, oligonucleotides, and proteins, for which HRMS can provide superior specificity and the potential of intact molecule analysis. There has also been considerable interest for application of alternative

ionization mode, fragmentation mode, and ion mobility for improved specificity, sensitivity, or the capability to analyze certain types of molecules/structure. Finally, MSI and other emerging surface MS analysis techniques have enabled direct visualization of molecules *in situ* in the tissue, which has brought new insight into biomarker research.

Great challenges and opportunities exist in many areas of LC–MS technologies in order to meet the ever-growing needs of better sensitivity, higher specificity, and higher-throughput analysis of biomarkers. In comparison with LBA, LC–MS generally give less sensitivity, particularly for HRMS analysis of large molecules. Advances in the areas such as sample preparation, ionization, and MS detection are expected to improve the capability of

LC–MS to quantify biomarkers at extremely low levels. Quantitation of large molecules at intact level has emerged as a valuable complement of the MRM-based “bottom-up” approach because of its capability to provide high level structure information. However, many hurdles still remain such as lack of sensitivity, specificity, and throughput. It is hoped that HRMS instrument equipped with better sensitivity, higher resolution, faster scan rate, and powerful software will eventually make it possible to routinely quantify large molecule biomarkers at intact level. Miniaturized instrumentation that can be operated *in situ* is another area of great interest and potential. It is expected that more efficient, real-time “bedside” clinical analysis will greatly facilitate diagnosis and treatment of disease and will be of great benefits to the patients.

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**5**

## **Comparison Between LC–MS and Ligand-Binding Assay Approaches for Biomarker Quantification**

*QingQing Wang, Lili Guo, and Ian A. Blair*

*Center of Excellence in Environmental Toxicology and Penn SRP Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA*

### **5.1 General Considerations: LBAs or LC–MS Assays**

Ligand-binding assays (LBAs) have been used for bioanalysis of small and large molecules since the late 1950s. The widespread usage of LBAs in biomarker analysis is attributed to their inherent high sensitivity and throughput, especially for large molecules presenting at femtomolar to attomolar levels in biological matrices (Zhang et al., 2014; Bults et al., 2015). Enzyme-linked immunosorbent assay (ELISA) is a typical format of LBAs, which is performed using a primary immobilized capture antibody, while the concentration of the targeted analyte is detected through the binding of secondary antibody coupled to enzymes, and the detectable signal is produced by the enzymatic conversion of substrate molecules. Sandwich ELISA is considered highly specific for targeted biomarker measurements because it uses two epitopes for detection rather than one. However, sandwich ELISA is less suitable for multiplexed measurements because of a greater incidence of nonspecific signals (Tighe et al., 2015; Chatziharalambous et al., 2016). In LBA measurements, it is important to choose appropriate antibodies since LBAs rely heavily on the quality of assay reagents. Unfortunately, in most cases of biomarker analysis, LBAs may not have the required specificity to distinguish the original compound from its metabolites (in small molecule biomarker analysis) (Haring et al., 2013) or distinguish intact protein from its heterogeneous forms (in protein biomarker analysis) (Yassine et al., 2013; Wang et al., 2015b). Therefore, the assay specificity and accuracy can be questionable if the antibodies do not possess suitable quality. Even after many years of improvement and optimization, developing highly specific antibodies for LBAs for a new target is still time consuming and expensive.

The aforementioned problems have prompted the development of alternative solutions that are in line

with the requirements for a specific, cost-effective, time-saving, and high-throughput analysis for biomarker discovery and validation. With the development of increasing sensitivity, mass spectrometer coupled with liquid chromatography (LC–MS) has become a useful alternative to LBAs for many bioanalytical applications. The success of LC–MS is mainly driven by its high specificity, broad dynamic range, fast method development, and the fact that it does not necessarily require immunochemical reagents (Chiu et al., 2009; Hoofnagle and Wener, 2009; Kitteringham et al., 2009; Ciccimaro and Blair, 2010) (Table 5.1). Among available LC–MS quantitative techniques, selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM), conducted with a triple quadrupole instrument, has been widely adopted as the “gold standard” for quantitative measurement (Ciccimaro and Blair, 2010; Yassine et al., 2013). In a typical LC–MRM analysis, which is also often referred to as LC–MS/MS analysis, a precursor ion is selected in the first quadrupole (Q1), then subjected to collision-induced dissociation (CID) in the second quadrupole (Q2), and the product ion(s) is selected in the third quadrupole (Q3). LC–MRM has evolved as a widely accepted, multiplex-capable method for quantitative determination of both small molecule and protein biomarkers. However, for protein biomarker analysis, LC–MRM method, if without coupling with immunoaffinity step, lags behind LBAs which have sensitivity at fg/mL level (Ackermann and Berna, 2007; Ackermann, 2012).

LBAs and LC–MS assays are both used in biomarker discovery and validation according to their particular strengths and weaknesses at different phases (Ackermann and Berna, 2007; Hoofnagle and Wener, 2009; Booth, 2011) (Table 5.1). Both quantitative platforms have played fundamental roles in biomarker discovery and verification phases, while LC–MS/MS is essentially used in the validation phase since biomarker validation requires a higher sensitivity and specificity for distinguishing

**Table 5.1** Pros/cons of LBA and LC-MS for biomarker analysis.

Attributes	Ligand-binding assay (LBA)	Liquid chromatography–mass spectrometry (LC–MS)
Throughput	High	Low
Cost	Inexpensive	Expensive
Method development	Slow	Fast
Dynamic range	<2 orders	>3 orders
Sample pretreatment	Simple No proteolytic digestion	Intensive Proteolytic digestion
Sensitivity	High	High sensitivity with expensive instrumentation
Specificity	Monospecific (antigen-antibody recognition) Low	Multispecific (retention time, precursor $m/z$ , product $m/z$ ) High
Precision	Moderate (CV < 25%)	Good (CV < 15%)
Affinity-dependent	Sensitive	Not relevant
Analyst-dependent	Yes	No

specific biomarkers of interest in complex matrices. Nevertheless, for quantification of targeted biomarkers, it is still challenging to translate the method from relative measurement to absolute quantitation using LBAs or LC–MS assays.

## 5.2 General Quantification Approaches

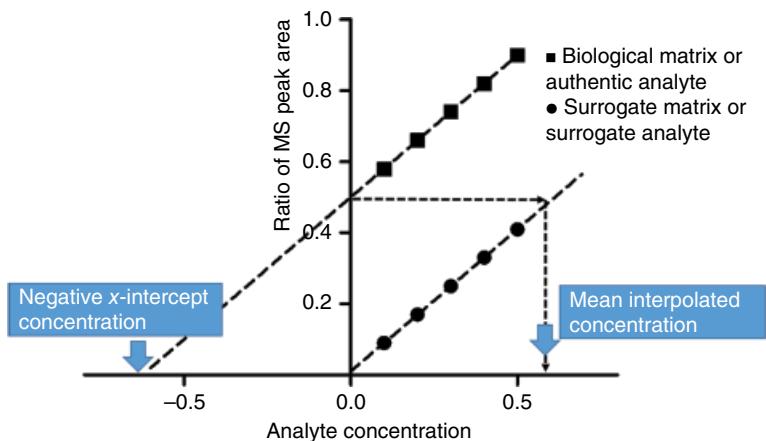
Three bioanalytical strategies have been developed to implement targeted quantification of biomarkers (Lee, 2009; Jian et al., 2012): authentic analyte in surrogate matrix, surrogate analyte in authentic matrix, and authentic analyte in authentic matrix. In general, it is not practicable to implement the strategy of “authentic analyte in authentic matrix” due to the difficulty in finding the analyte-free matrix for biomarker analysis. Besides, for protein biomarker quantification, it is very challenging to find the protein analyte for use as a calibrator.

In the surrogate matrix approach, a substitute to the biological matrix that is devoid of the analyte is used for the creation of the calibrators. This may be a synthetic mixture (e.g., artificial cerebral spinal fluid (Oe et al., 2006; Hooshfar et al., 2016)), an analyte-depleted matrix (e.g., charcoal-stripped matrix (Rangiah et al., 2011; Wang et al., 2015a)), or a biological matrix from an alternate species (Thway and Salimi-Moosavi, 2014; Wang et al., 2015b). For example, in the case of analysis of estrogen and its metabolites in human serum, double charcoal-stripped male serum was used because the endogenous estrogens in it were below the lower limit of quantification (LLOQ) (Rangiah et al., 2011; Wang

et al., 2015a). For protein/peptide biomarker analysis, a biological matrix from nonhuman species can be employed when the sequence of the protein/peptide biomarker is not fully conserved across species. An example is apolipoprotein A1 (ApoA1) quantification in human serum, where mouse serum was used to prepare the calibration curve and QC samples because all tyrosine-containing peptides differ from corresponding peptides in mouse ApoA1 by at least one amino acid (Wang et al., 2015b). However, some confounding factors should be taken into consideration when this approach is employed. Solubility, matrix effect, specificity, and/or recovery of target analytes in surrogate matrix may not be truly comparable to what would be seen in authentic matrix due to the different nature of the surrogate matrix from the authentic one. Therefore, it is advisable to prepare QC samples in the true biological matrix in order to fully address those potential issues when using this surrogate matrix approach (Ho and Gao, 2015; Suhr et al., 2016).

For the surrogate analyte approach, a stable isotope-labeled (SIL) analog of the targeted biomarker is used in place of the authentic analyte. A calibration curve is generated with this SIL analog, and the calculation of biomarker concentration is based on the regression equation from this analog curve. Because the SIL has physical and chemical properties nearly identical to the target biomarker but it is not present in the matrix, calibration curve and QC samples can be prepared in the intended biological matrices (Jian et al., 2012; Jones et al., 2012). Unlike stable isotope dilution (SID) method, the SIL analog is not added to every standard and sample, but instead it is spiked into only the calibration curve. Ideally, a second SIL analog is recommended for inclusion as an internal standard (INSTD) (Jian et al., 2012; Liu et al., 2016).

**Figure 5.1** Conceptual illustration of the use of standard addition to assess parallelism with surrogate analyte and surrogate matrix using MS methods.



A correction factor should be employed in order to compensate for any potential response difference caused by an isotopic effect or change in ionization efficiency between the biomarker of interest and its SIL analog (Jemal et al., 2003; Jian et al., 2010). An interesting application is analysis of creatinine in human urine (Leonard et al., 2014). In this assay, [<sup>2</sup>H<sub>3</sub>]-creatinine was used as a surrogate analyte to quantify endogenous creatinine. However, since endogenous concentrations of creatinine were orders of magnitude higher than [<sup>2</sup>H<sub>3</sub>]-creatinine, the peak area of a less intense isotope of creatinine was acquired and a response factor was used to correct for using a less intense isotope MRM transition.

For both of aforementioned approaches, parallelism should be evaluated to ensure accurate quantitation. To date, most evaluations of parallelism in biomarker assays were performed during method development and pre-study validation phases (Stevenson and Purushothama, 2014). In LBA, the assessment of parallelism is similar to assessment of dilution linearity study. The major difference is that dilution linearity employs a spiked control sample with a known quantity of analyte, while parallelism employs an incurred sample and/or samples containing endogenous analyte (Ciotti et al., 2013; Stevenson and Purushothama, 2014). The dilution buffer and biological matrix are considered to be parallel if the signal decreases in accordance with the dilution factor. Validation of spike recovery for a targeted biomarker in a biological matrix is another way to assess parallelism for both LBA and LC-MS assay. The spike recovery is calculated by the following formula by comparing the concentrations of analyte in biological matrix and in surrogate matrix, where bm = biological matrix and sm = surrogate matrix:

$$\% \text{ Recovery} = \frac{(\text{Spiked bm} - \text{blank bm})}{(\text{Spiked sm} - \text{blank sm})} \times 100$$

The standard addition is another way to assess parallelism in surrogate analyte approach or surrogate matrix

approach in LC-MS methods (Jones et al., 2012) (Figure 5.1). In this method, when the endogenous concentration in biological matrix derived from the standard addition agrees with the concentration obtained by direct interpolation from the surrogate matrix or surrogate analyte, parallelism exists. This approach was demonstrated by Jones et al. (2012) in the analysis of five amino acids in human plasma including alanine, valine, methionine, leucine, and isoleucine where 0.2% bovine serum albumin in phosphate-buffered saline was used as surrogate matrix, and SIL analogs of five amino acids ([<sup>13</sup>C<sub>1</sub> <sup>15</sup>N<sub>1</sub>]<sub>L</sub>-alanine, [<sup>13</sup>C<sub>1</sub> <sup>2</sup>H<sub>3</sub>]<sub>L</sub>-methionine, [<sup>2</sup>H<sub>8</sub>]<sub>L</sub>-valine, [<sup>13</sup>C<sub>6</sub> <sup>15</sup>N<sub>1</sub>]<sub>L</sub>-leucine, and [<sup>13</sup>C<sub>6</sub> <sup>15</sup>N<sub>1</sub>]<sub>L</sub>-isoleucine) were used as surrogate analytes. The author showed both approaches were well within acceptance criteria prescribed by regulatory guidance for method validation. When comparing the two approaches, the surrogate analyte allows for easier method development when SIL INSTDs are readily available, while the surrogate matrix method requires greater up-front method development; however, this deficit is offset by simplifying sample analysis.

### 5.3 Analytical Issues Specifically Related to LBAs

#### 5.3.1 There Is No Sample Pretreatment in Most LBAs

Different from LC-MS methodologies, LBA protocols seldom include sample pretreatment since the pretreatment step might alter conformation or binding activity between antibody and antigen. The complexity of the biological matrix generally is removed or minimized by sample dilution in buffers, a process called minimal required dilution (MRD) (Gorovits et al., 2014; Wang and Ma, 2014). Therefore, evaluation of matrix effect and minimizing matrix interference are critical issues in LBAs.

The matrix effect in LBAs is often observed in the early method development stage as a discrepancy between standard curves in buffer and in biological matrix. The evaluation of matrix effect for biomarker analysis is usually evaluated by comparing spike recovery in biological matrix and surrogate matrix (as described in Part 2). Which type of surrogate matrix is suitable is decided by the baseline level of biomarker of interest and specific study purpose. For example, in the quantification of hepcidin in human serum by ELISA (Thway and Salimi-Moosavi, 2014), the matrix effect can be evaluated with rabbit serum because human hepcidin does not cross-react with rabbit hepcidin. While in the case of sclerostin, the evaluation of matrix effect can be tested only in substituted matrix (protein buffer) because antibodies raised against human sclerostin cross-react with multiple species.

There are many conventional approaches for minimizing the matrix interference in protein biomarker analysis. Optimization of assay reagent concentrations and utilization of assay buffers with salt, protein, or detergent have been used to reduce nonspecific binding. For example, in the quantification of tumor necrosis factor alpha by ELISA in human serum (Karakus et al., 2005), dilution buffer (1% bovine serum albumin in PBS) with a certain percentage of saturated ammonium sulfate (9%) was shown to amplify the signal and eliminate the endogenous interference of human serum. In some cases of measurement of small molecule biomarkers, samples can be pretreated by liquid–liquid extraction (LLE), solid-phase extraction (SPE), or chromatography before loading on the plates. In an example of analysis of testosterone in human serum or urine, radioimmunoassays (RIAs) incorporating extraction and chromatography showed greater sensitivity compared to the method without extraction (Stanczyk, 2004).

### **5.3.2 It Is Hard to Distinguish Biomarkers and Their Variants by LBAs**

LBAs are based on the ability to recognize the analyte from complex matrix using highly specific reagents. Paradoxically, many disease-specific biomarkers are anticipated to be present at very low levels (ng/mL or lower). Therefore, LBAs may not have enough specificity to measure specific biomarker of interest from gigantic interferences because it is hard to generate binding/capture reagents for unique epitopes only on intact biomarkers. Cross-reaction from the isobaric or structurally similar molecules (in small molecule biomarker analysis) or heterogeneous forms (in protein biomarker analysis) may cause overestimation. Take the analysis of 8-iso prostaglandin F<sub>8,2α</sub> as an example (Tsikas et al., 2003; Smith et al., 2011). Theoretically, the F2-isoprostane

family consists of 64 different isomers. ELISA data normally showed higher values than LC–MS and GC–MS, which may be due to cross-reactivity of the antibodies with other isoprostane isomers or metabolites.

Differential LBA methods recently have been investigated to distinguish between free and total forms (Lee et al., 2011), and differentiate heterogeneity of protein biomarkers (Diamandi et al., 2000). Diamandi et al. reported an interesting study showing that LBAs are capable of differentially determining variants of insulin-like growth factor-binding protein 3 (IGFBP-3) in various biological fluids (serum, seminal plasma, amniotic fluids), pregnancy serum, and breast tumor cytosol (Diamandi et al., 2000). Based on evaluation of a panel of monoclonal and polyclonal IGFBP-3 antibodies, three ELISAs were constructed using a common capture and polyclonal (ELISA-3) or monoclonal (ELISA-1 and -2) detection antibodies. Evaluation of IGFBP-3 after proteolysis suggested recognition of intact IGFBP-3 by ELISA-1, whereas ELISA-3 appeared to measure intact and proteolyzed IGFBP-3 (total IGFBP-3) with similar potency. In contrast, the preferential recognition of IGFBP-3 fragments was shown by ELISA-2. Up to 19-fold variation was observed when comparing the values obtained by this study to corresponding levels by an established ELISA kit (Diagnostic Systems Laboratories Inc. active IGFBP-3 ELISA). However, not all of the heterogeneous forms can be easily differentiated by LBAs. In the study of quantification of ApoA1 in human serum, Wang et al. (2015b) showed LC–MS/MS could discriminate minor ApoA1 changes between smokers and non-smokers, which were not revealed by an ELISA kit (Abcam, Catalog Number ab108804). This minor difference in ApoA1 may be due to unknown post-transitional modifications on some tryptic peptides, as three of eight tyrosine-containing peptides did not show good correlations with other peptides.

## **5.4 Analytical Features Specifically Related to LC–MS Methods**

Because of the revolutionary advances in mass spectrometry technologies, the quantification of small molecular biomarkers mostly has shifted to the use of LC–MS/MS or LC-high-resolution/accurate mass spectrometer (HRMS). Over time, with the advances in proteomics, MS technologies have made a splash in the protein biomarker analysis. In order to achieve the best accuracy and specificity, LC–MS, in combination with SIL INSTD, has been extensively investigated in the quantification of both small molecule and protein biomarkers.

### 5.4.1 Proper Sample Preparation Generates Better Data

Compared to protein biomarker analysis by LC-MS, the sample preparation for small molecule biomarkers is relatively straightforward. It typically involves solvent precipitation, LLE, SPE, and immunoaffinity depletion. Cleaning up interferences from large molecules and concentration of analytes are two main purposes of this step. The optimization of extraction solvents, SPE cartridges, and immunoaffinity conditions is essential. An extra derivatization approach can be employed if the biomarker of interest is at extremely low concentration (Zhu et al., 2015). For example, the typical serum concentration range of unconjugated estradiol (E2) in postmenopausal women is only 2.7–15.9 pg/mL (Wang et al., 2016). This concentration range is still challenging for most LC-MS-based assays. With derivatization technology, especially by giving permanent charge to the analytes (pre-ionization), the detection limit of LC-MS was able to reach fg/mL, which allows accurate absolute quantification of estrogen and its metabolites in prepubertal children, postmenopausal women, and elder men (Blair, 2010; Kushnir et al., 2011).

Quantification of protein biomarkers using LC-MS methodology has its own unique complexities. Some sample preparation processes used in small molecule biomarker analysis are also used, such as removing the high abundance proteins (HAP) by solvent precipitation or immunoaffinity depletion, the latter of which has been demonstrated for protein analysis in several studies (Prieto et al., 2014; Filip et al., 2015). The reproducibility of immunoaffinity depletion was showed in a large-scale interlaboratory study, where 11 laboratories, using multiplexed MRM assays on 14 LC-MS systems, precisely and reproducibly measured 125 peptides derived from 27 cancer-relevant proteins and 7 control proteins (Addona et al., 2009; Abbatiello et al., 2015). The depletion of the top 14 most abundant human plasma proteins decreased the sample complexity and allowed an effective concentration of analytes to be loaded onto the column. The limits of quantification (LOQs) were improved three- to five-fold (at peptide level), while the assay multiplex level was simultaneously increased more than 10-fold. One should be aware that while immunoaffinity depletion columns may deplete high-abundance plasma proteins efficiently, they could potentially also remove protein biomarkers of interest either due to protein–antibody binding or due to other interactions between protein analyte and column (Gundry et al., 2007).

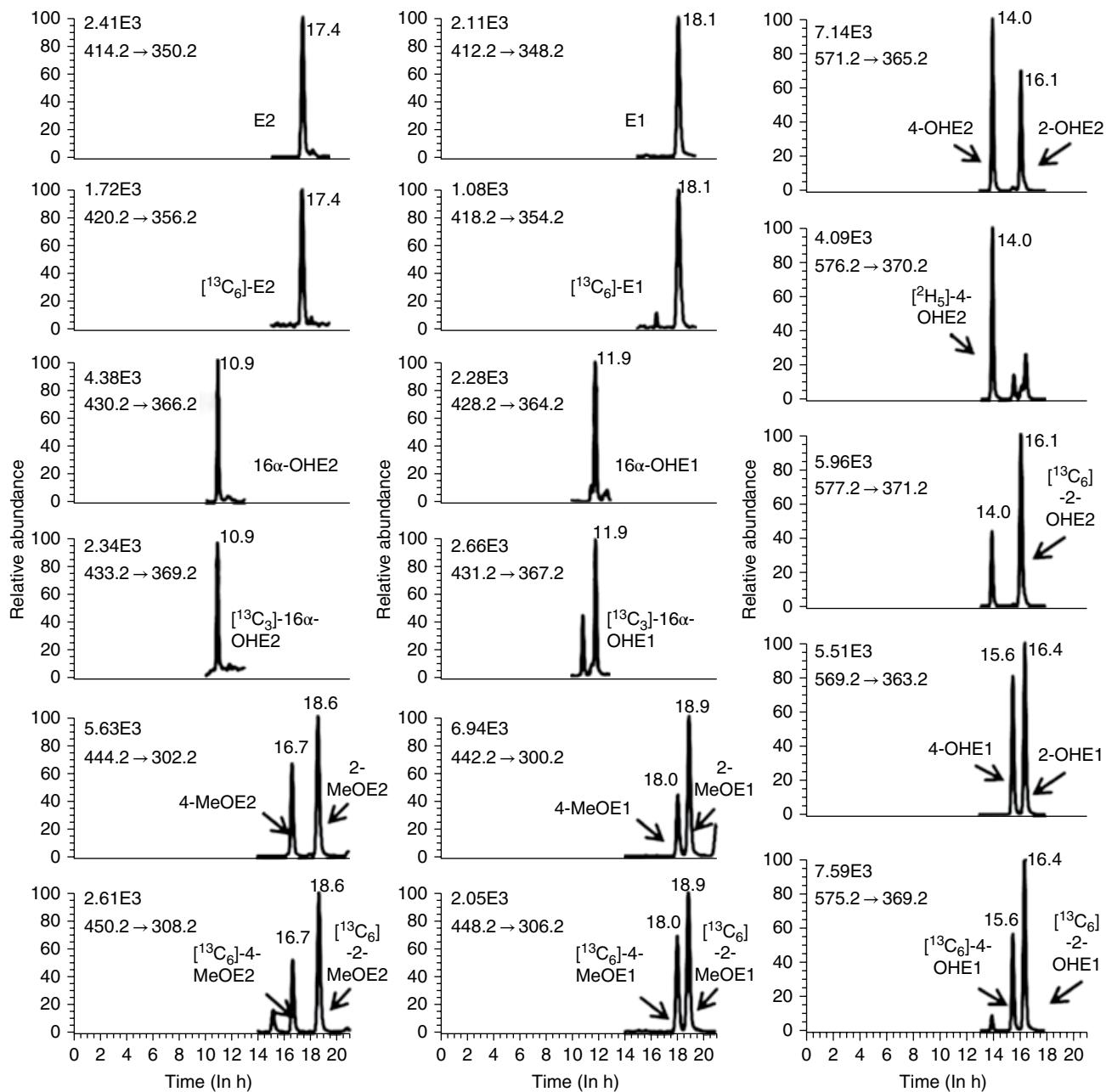
The common enrichment methods conducted for protein biomarkers include the use of antibodies (immunoaffinity capture), gel electrophoresis, or off-gel fractionation. Enrichment by immunoaffinity capture

was demonstrated in an LC-MS/MS assay for quantification of growth and differentiation factor 8 in serum (GDF-8) (Palandra et al., 2016). The workflow included the enrichment of GDF-8 using an anti-GDF-8 antibody, followed by isolation using magnetic beads, trypsin digestion, and quantification using 2D nanoflow LC-MS/MS. This assay was qualified in human serum with an LOQ of 1.0 ng/mL based on the intact protein.

Recently, a new approach termed stable isotope standards and capture by antipeptide antibodies (SISCAPA) was introduced into protein biomarker analysis (Anderson et al., 2004). The peptides of interest are enriched from digested samples along with their SIL peptides by antipeptide antibodies on immunoaffinity column or magnetic beads. After wash steps, the peptides are released and quantified by LC-MS/MS. Many studies have shown significant potential of SISCAPA approach for verification and quantification of low abundance (pg/mL) protein biomarkers in plasma (Whiteaker et al., 2011; Razavi et al., 2012). One advantage of SISCAPA over the approach with enrichment at protein level is that auto-antibodies toward tumor antigen (as biomarkers) can be measured more effectively by SISCAPA assay. As a result of the enzymatic sample processing, the auto-antibodies are no longer masked by the tumor antigens as it would be in the immunoaffinity process at protein level. Thus the assay is less prone to producing false-negative results (Kuhn et al., 2012).

### 5.4.2 Biomarkers and Their Variants Can Be Distinguished

The greatest challenge for quantification of small molecule biomarkers comes from the difficulty in differentiating biomarker itself from isobaric compounds or structural analogs. Specificity under SRM or MRM mode is gained by monitoring product ions fragmented from the selected precursor ion(s). However, in complex biological matrices, the selectivity of unit resolution mass spectrometer is limited by the resolving power of its mass analyzers, and interferences may cause biased results. Steroid hormone analysis represents a good case. For example, 17-hydroxy-progesterone (OPH) and deoxy-corticosterone (21-OHP) are indistinguishable in a triple quadrupole (QQQ) mass spectrometer due to their identical MRM transitions (Vogeser and Seger, 2010). It is also challenging for quantification of 2- and 4-hydroxy-estrogens and their corresponding methoxy-metabolites on a QQQ mass spectrometer because each pair of isomers has identical MRM transitions and must be chromatographically separated from each other (Wang et al., 2016) (Figure 5.2). The practical approach to avoid the isobaric interference is to maintain a good chromatographic separation. The advantages of HRMS



**Figure 5.2** LC-SRM chromatograms for analysis of estrogens and their metabolites extracted from double charcoal-stripped human serum as pyridinium sulfonyl (PS) derivatives. Source: Wang et al. (2016). Reproduced with permission of Elsevier.

in the quantification of small molecule analysis have been shown in some studies (Ryu et al., 2015). Even for LC-HRMS assays, it is desirable to chromatographically separate the inferences in order to achieve selectivity for complex samples. In the hybrid mode (LC-MS/HRMS), the fragment ion chromatograms can also be extracted using a narrow mass tolerance (<5 ppm) to ensure assay specificity.

The quantification of protein biomarkers by LC-MS/MS is normally performed by digesting proteins followed by analysis of one or more surrogate peptide(s). The concentrations of protein biomarkers were achieved by measuring the ratio of the surrogate peptide(s) and INSTD peptide(s). Therefore, a prerequisite of LC-MS/MS for quantifying protein biomarkers is that the surrogate peptides and their optimal specific fragmentation

transitions must be known. For example, in the quantification of apolipoprotein A1 in human serum (Wang et al., 2015b), methionine oxidation was discovered and measured by adding an extra ion transition [ $M + 16$ ] on the peptide WQEEMELYR. The absolute quantification of this peptide was based on total methionine (nonoxidized M + oxidized M).

Protein biomarkers may have post-transitional modification, and protein metabolism may cause glycosylation, phosphorylation, oxidation, and deamidation (Johnson and Evers, 2010). There are many chemically induced modifications on some active amino acids of protein side chains (Ahmed and Thornalley, 2003; Liu et al., 2013). Moreover, due to biological interactions, a protein biomarker can exist in different forms (free, partially bound, bound) with distinct physiological functions (Lee et al., 2011). Therefore, there are two essential steps for the quantification of a protein biomarker: identification of the exact molecular form of the biomarker in the biological matrix, as well as quantification of its concentration in the matrix (Christenson and Duh, 2012). One should be alert to potential bias inherent in protein quantification via LC-MS/MS methodology, since the quantification depends on the physicochemical and analytical properties of the selected surrogate peptides. The uniqueness of peptides should be confirmed to ensure selected peptides are only derived from the targeted protein instead of other proteins in the biological matrix. Correlation analysis with concentrations measured by other surrogate peptides from the same protein will help to determine potential modifications, as well as increase the confidence of intact protein quantification. Another technology used to rapidly identify protein heterogeneity is mass spectrometric immunoassay (MSIA). MSIA is based on the isolation of proteins by immobilized antibodies, followed by direct intact MS analysis rather than LC-MS/MS. When immunoaffinity approach is employed to isolate intact proteins, most single amino acid and post-translationally modified variants can be readily determined and quantified (Yassine et al., 2013). However, like antibody-based assays (LBAs), the selectivity and specificity of MSIA are limited by the availability of high-quality antibodies.

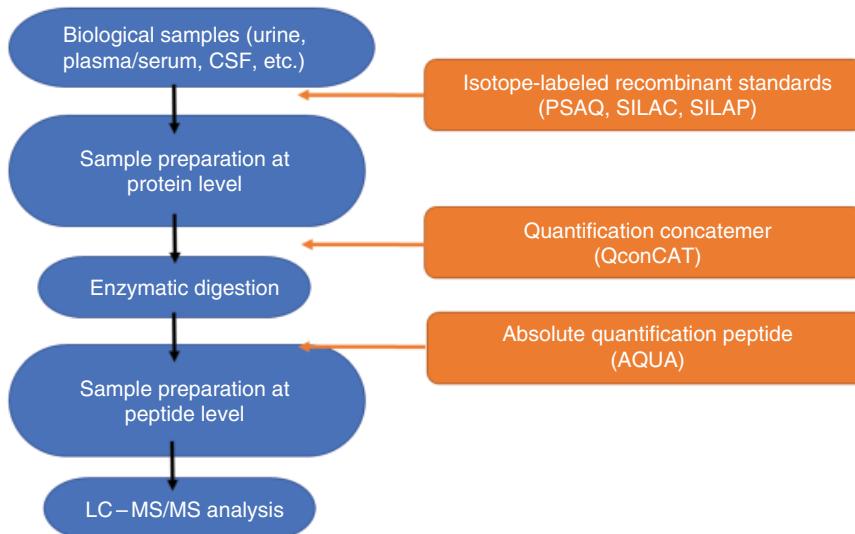
#### **5.4.3 Stable Isotope-Labeled Internal Standard Used for Assuring the Assay Accuracy**

Absolutely accurate quantification by LC-MS can be achieved by using SIL analogs for both small molecule biomarkers and protein biomarkers. SIL compound is spiked in all samples as INSTD to compensate experimental variability occurring during sample preparation, proteolytic digestion, and ionization. For small molecule

biomarker analysis, choosing a suitable SIL INSTD is quite straightforward. Careful considerations should be taken in deciding which SIL compound is used as INSTD when there are more than one SIL compound. In general, extensively deuterated form of a compound is not a perfect INSTD since there is a potential chromatographic separation of deuterated compound and its corresponding analyte. Ideally, [ $^{13}\text{C}$ ]-labeled INSTD should be used because they co-elute with the nonlabeled version under all chromatographic conditions. However, for many biomarkers, only deuterated or structural analogs are available. Take estrogen as an example; there is only [ $^2\text{H}_5$ ]-4-hydroxy-estradiol available (Blair, 2010; Wang et al., 2016).

Choosing of SIL INSTD is more complicated for protein biomarker analysis. Typically, isotopically labeled tryptic peptide(s) specific to the protein of interest is used as internal standard(s), a strategy known as absolute quantification (AQUA) (Brun et al., 2009). In the AQUA approach, peptide INSTDs are normally introduced to the protein sample after proteolytic digestion (Figure 5.3). The commercial availability and wide choices of AQUA peptides make this approach particularly attractive. The AQUA approach is based on the same principle as the classic SID approach for quantification of small molecules; the critical difference is that quantification of protein by AQUA analysis relies on measurements of surrogate peptides. The reproducibility of protein prefractionation and efficiency of peptide released from protein are particularly important for the quantification at protein level (van den Broek et al., 2013). Therefore, verifications of completeness of target protein digestion and stability of surrogate peptides are recommended when employing the AQUA approach (Brun et al., 2009; Kettenbach et al., 2011). Moreover, if a prefractionation step precedes tryptic digestion, the recovery yield of target protein should be evaluated.

In contrast to AQUA peptides, which are generated by chemical synthesis, quantification concatemer (QconCat) peptides are derived from artificial protein(s). The artificial protein, QconCat protein, is a concatemer of proteolytic signature peptides generated by gene design *de novo*. QconCat proteins were reported to be highly amenable to proteolytic digestion due to their lack of folding (Beynon et al., 2005; Pratt et al., 2006). QconCat protein is typically added in biological samples before the digestion step (Figure 5.3), so it partially compensates for digestion differences among different samples. However, QconCat protein may not completely track the digestion efficiency of the target protein due to its structural differences with the analyte. To control all the variables from protein prefractionation to LC-MS/MS analysis, an isotope-labeled equivalent of the full-length target



**Figure 5.3** Introduction points of different INSTDs for quantification of protein biomarkers.

protein standard is preferred. The Brun's group (Brun et al., 2007) proved this concept by demonstrating that isotope-labeled protein standard (PSAQ) showed better accuracy than AQUA peptides and QconCat protein for quantifying the biomarkers of staphylococcus superantigenic toxins in urine samples. PSAQ strategy was also coupled with immunoaffinity-based nano-LC–MS/MS for the quantification of *Staphylococcus aureus* enterotoxin A in serum and showed enhanced sensitivity and specificity (Adrait et al., 2012).

Unfortunately, PSAQ proteins are typically expressed by cell-free biosynthesis systems or bacteria and do not carry post-translational modifications, while numerous biomarkers belong to the tissue-leakage protein family and normally bear labile modifications. Ultimately, two new strategies were introduced to circumvent this problem and extend the PSAQ applications: stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002; Ong and Mann, 2006) and stable isotope labeling proteomics (SILAP) (Yan et al., 2005). In a typical SILAC/SILAP workflow, an immortalized cell line is grown in media containing either unlabeled essential amino acids or their labeled counterparts. The unlabeled and labeled cells are then treated with either a control or experimental condition, followed by mixing and a bottom-up LC–MS analysis to reveal the difference between the two treatment groups. In most recent spike-in SILAC approaches, SILAC is only used to produce heavy labeled proteins or proteomes as standard for absolute quantitation or identification of biomarkers (MacDonald et al., 2012; Wang et al., 2015b) (Figure 5.3). SILAC standard can be expressed in mammalian cell lines (e.g., HEK293, Hela, HepG2) facilitating both in-depth identification and absolute quantification of biomarkers. In an innovative

study by Harel et al. (2015), a proteomics method was developed employing microparticles with super-SILAC quantification, which enabled relative and absolute SILAC quantification.

## 5.5 Case Studies: Comparison Between ELISA and LC–MS

### 5.5.1 Steroid Analysis

Steroids were measured by RIA, ELISA, and LC–MS/MS (Table 5.2). In a few cases, LBA measurements (RIA and ELISA, automated or manual) of steroid metabolites in urine or serum correlated well with LC–MS/MS analysis (Wang et al., 2004; Faupel-Badger et al., 2010; Haring et al., 2013). The higher concentrations steroids were, the better correlations were observed. For example, in the analysis of estrogen and its metabolites (estrone, estradiol, estriol) (Faupel-Badger et al., 2010), LC–MS/MS measurements were highly correlated with RIA or ELISA measurements in premenopausal women (Spearman  $r=0.8\text{--}0.9$ ) and moderately correlated (Spearman  $r=0.6\text{--}0.67$ ) in postmenopausal women. The concentrations of 2-hydroxy-estrone and 16 $\alpha$ -hydroxy-estrone by LC–MS/MS showed weak correlations with RIA and ELISA measurements (Spearman  $r=0.2$ ). The absolute concentrations measured by LBAs were usually higher than those obtained by LC–MS/MS. In postmenopausal women, the concentration of 16 $\alpha$ -hydroxy-estrone using ELISA was 12 times higher than that measured by LC–MS/MS (Faupel-Badger et al., 2010). The reason for overestimation by LBAs could be the cross-reactivity with other steroid metabolites.

**Table 5.2** Typical studies of quantification of steroids by LBAs and LC–MS assays for the same samples.

Biomarker	Reference	Biofluid	Ligand-binding assay (LBA)		Liquid chromatography-mass spectrometry (LC-MS)	Comparison (Pearson or Spearman correlation analysis)	Bias (mean values analysis, Bland-Altman analysis)
			Assay format	Critical reagents			
Total testosterone	Wang et al. (2004)	Serum	Automated immunoassay (RIA)	Roche Elecsys, Bayer Centaur, Ortho Vitros Eci, DPC Immulite 2000	API 3000 (AB)	Deming regression: Roche Elecsys (1.167), Bayer Centaur (1.195), Ortho Vitros Eci (1.233), DCP Immulite (0.881)	Roche Elecsys, ortho Vitros Eci and DPC Immulite showed negative bias; Bayer Centaur showed positive bias
			Manual immunoassay (RIA)	DPC-RIA, HUMC-RIA		Deming regression: DPC-RIA (1.098), HUMC-RIA (1.141)	
Total estrogen (estrone, estradiol, estriol)	Faupel-Badger et al. (2010)	Urine	RIA	Quest Diagnostics (Nichols Institute)	TSQ Quantum-AM (ThermoFinnigan)	Spearman rank correlation: E1 (0.79–0.94), E2 (0.63–0.91), E3 (0.73–0.94)	RIA showed positive bias
Total estrogen (2-hydroxyestrone, 16α-hydroxyestrone)			ELISA	Improved kit (Immunacare)		Spearman rank correlation: 2-OH-E1 (0.37–0.81), 16α-OH-E1 (0.62–0.86)	ELISA showed positive bias
Total testosterone	Haring et al. (2013)	Serum	Chemiluminescent immunoassay	Immulite 2500 analyzer (Siemens Healthcare Medical Diagnostics)	Quattro Micro tandem MS (Waters)	Pearson correlation: $r=0.84$ , $p<0.05$	

ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay.

### 5.5.2 Apolipoprotein A1

Apolipoprotein A1 levels were quantified by ELISA, immunoturbidity assay, and LC-MS/MS in human plasma and nonhuman primate plasma. In the study where one or two surrogate peptides were measured by AQUA LC-MS/MS method, high correlation ( $R > 0.9$ ) with ELISA was obtained (van den Broek et al., 2016). However, in Wang's study (Wang et al., 2015b) where nine surrogate peptides were used to quantify apolipoprotein A1 level in human plasma by a SILAC approach, poor correlations with ELISA in both smokers (slope = 0.32 by Deming regression) and nonsmokers (slope = 0.25 by Deming regression) were observed. One reason for these discrepancies may be due to calculation of protein levels based on different peptides, for which the tryptic efficiency and stability were highly variable. Another possibility was that the introduction points of INSTDs in these studies were different—AQUA peptides were introduced into plasma sample after tryptic digestion, while SILAC peptides were spiked at the beginning of sample preparation.

## 5.6 Summary and Future Perspective

The quantitative analysis of biomarkers has been playing an ever-increasing role from discovery-based quantitative process to verification-based quantification in clinical and preclinical settings. While there is no clear regulatory recommendation to guide the quantitative analysis of biomarkers, a fit-for-purpose approach should be used for biomarker quantification at different stages of biomarker discovery and validation. Due to the

endogenous nature, the analysis of both small molecule and protein biomarker presents a series of challenges for assay development and validation. LBAs and LC-MS assays share some common approaches to resolve these endogenous nature-related issues. One should keep in mind that the validation of assay selectivity and specificity is crucial when employing LBAs for biomarker quantification since LBAs highly depend on the quality of antibodies. As the continuous development of mass spectrometers and chromatography columns, it is conceivable that LC-MS/MS will play an increasing role in biomarker analysis to support clinical studies and mechanism-based researches. Discrimination of metabolites (for small molecule biomarkers) and heterogeneity (for protein biomarkers) requires comprehensive considerations in LC-MS/MS assays. SIL analog offers a means to verify the presence of analyte and compensate experimental varieties which can provide absolute quantification.

In recent years, LBAs have been improved in many innovative ways. Gyrolab immunoassays provide the advantage of higher throughput and substantially reduced reagent consumption by combining the microfluidic method and fluorescence detection. Luminex- and MSD-based assays allow the multiplex analysis with high sensitivity. At the same time, high-resolution/accurate mass spectrometers have been applied to the development of more comprehensive analysis in metabolites and protein heterogeneity. An interesting trend has emerged in biomarker analysis which blends LBA and LC-MS techniques to combine the selectivity of immunoaffinity extraction with the specificity of MS detection. It is a very attractive perspective that integration of ligand-binding and LC-MS assays will help provide more meaningful data for biomarker quantification.

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## 6

# Sample Preparation Methods for Targeted Biomarker Quantification by LC-MS

Shichen Shen<sup>1,2</sup>, Bo An<sup>2,3</sup>, and Jun Qu<sup>2,3</sup>

<sup>1</sup> Department of Biochemistry, Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, NY, USA

<sup>2</sup> New York State Center of Excellence in Bioinformatics and Life Sciences, Buffalo, NY, USA

<sup>3</sup> Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, Buffalo, NY, USA

## 6.1 Introduction

Sample preparation is an essential component for bioanalytical methodologies (Kole et al., 2011). Biomarkers of interest often present at low concentrations in biological matrices (e.g., tissues, serum, and plasma) with complex compositions and possibly high dynamic range (Anderson et al., 2004a), which led to considerable challenges for the LC-MS analysis. For example, endogenous substances (e.g., salts, phospholipids, nucleic acids, sugars, and peptides) co-eluting with the biomarker could severely compromise sensitivity, reproducibility, and robustness of the LC-MS analysis, if the sample was not cleaned up effectively (Bylda et al., 2014). The ultimate goals for sample preparation in LC-MS-based biomarker quantification are therefore three-fold: (i) when needed, to extract and solubilize analytes into a solution and render the sample suitable for LC-MS analysis; (ii) to enrich the target analyte (i.e., biomarker) and remove interfering components in the matrix, reinforcing sensitivity, selectivity, and robustness of the assay; (iii) to retrieve biologically meaningful information that may be inaccessible by using LC-MS approaches alone (e.g., subcellular localization, protein-ligand interaction, protein biotransformation). In practice, sample preparation procedures are usually the most time-consuming and labor-intensive step of the overall analytical process and are susceptible to errors and variability (Fu et al., 2005; Kole et al., 2011; Pavlović et al., 2007).

Depending on the types of biomarkers to be quantified, sample preparation strategies vary substantially. For small molecule biomarkers (e.g., amino acids, oligopeptides), the target analyte is already present in the biological matrix, so strategies such as protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE) are usually used to address the

needs of target enrichment and interference removal under most circumstances. For macromolecule biomarkers (e.g., proteins), however, more sophisticated sample preparation procedures are usually demanded. Biological samples need to be pretreated and proteins are denatured with reactive cysteine residues shielded and then proteolytically digested to derive surrogate peptides or signature peptides (SPs) to be analyzed in LC-MS (Gillette and Carr, 2013). While sample preparation strategies for small molecules may also apply for macromolecules, additional approaches such as sample fractionation, depletion of high abundance matrix proteins, and immunoaffinity enrichment of target proteins or proteolytic peptides are also performed as an option to further boost the selectivity and efficiency of sample preparation procedures. Finally, the detailed sample preparation methods are also dependent on specific analytes based on their unique physicochemical properties.

In this chapter, we elaborate and discuss the current methodologies of sample preparation for both small molecules and macromolecules, with an emphasis on issues of sensitivity, selectivity, and robustness. Several common issues will be discussed first, followed by the rationale, features, and limitations of individual methods. Applications of the methods are also illustrated.

## 6.2 Sample Preparation Strategies for Small Molecule Biomarkers

Small molecule biomarkers include (and are not limited to) sugars, lipids, sterols, nucleic acids, amino acids, and oligopeptides. These biomarkers could be in the free form but often are sequestered by protein molecules in biological matrices, which need to be removed to minimize matrix effects. Current sample preparation

**Table 6.1** Common sample preparation methods for small molecule biomarkers and their specifications.

Techniques	Selectivity	Sample cleanup	Analyte enrichment	Throughput	Costs	Applicable biomatrices	Novel variations
Dilute-and-shoot	-	-	-	+++	+	Urine	N/A
Protein precipitation (PPT)	-	+	-	++	++	Whole blood, plasma, serum	PPT plates, SPE-PPT
Liquid–liquid extraction (LLE)	++	++	+	+	++	Urine, plasma, serum, oral fluids	SALLE, SIPTE, SLE
Solid-phase extraction (SPE)	+++	+++	++	++	+++	Urine, whole blood, plasma, serum, oral fluid	SPE-PPT, SPME, SBSE, MEPS

strategies for small molecule biomarkers have been summarized in Table 6.1. Several popular methods (e.g., PPT, LLE, SPE) are described as follows. Other novel methods (e.g., magnetic particles and nanoparticles, turboflow, carbon nanotubes, RAM, immunosorbents, molecular imprinted polymers, and aptamers) with general applications are discussed in two recent reviews (Bylda et al., 2014).

### 6.2.1 Primary Issues to Address for Sample Preparation

#### 6.2.1.1 Matrix Effects

Matrix effects persist to be one of the primary challenges for LC-MS-based biomarker quantification, which refers to the detrimental effects that matrix components co-eluting with target molecules impose on the detection of the target (Kebarle and Tang, 1993). These matrix components, both endogenous (e.g., lipids, phospholipids, fatty acids) and exogenous (e.g., surfactants, ion-pairing reagents), could lead to ion suppression (under most circumstances) or ion enhancement effect and cause significant changes in the signal responses of the target molecule when analyzed in complex biological matrices compared with neat solutions (Bylda et al., 2014). The sensitivity, accuracy, and robustness of the analysis could be severely deteriorated if matrix effects are not properly addressed.

Mechanistically, matrix effects are thought to occur during ionization. Electrospray ionization (ESI) (Ho et al., 2003), especially, is highly vulnerable to matrix effects (King et al., 2000). One hypothesis for ESI-oriented matrix effects considers increases of droplet surface tension when interfering substances co-elute with the target molecule, which impedes solvent evaporation and the ability of target molecules to enter gas phase (Antignac et al., 2005; Jessome and Volmer, 2006) (Figure 6.1). This hypothesis explains why phospholipids and surfactants should be avoided or depleted during

sample preparation (Janusch et al., 2013). Another hypothesis is based on previous observation that ESI responses usually become nonlinear at higher analyte concentration (e.g.  $>10^{-5}$  M) due to the insufficient number of charges available on droplets or the inhibition of ion ejection at the droplet center caused by saturation of analytes on droplet surface. Since interfering substances increase the total “analyte” concentration, the ionization efficiency of the target molecule will be compromised, especially when the interfering substances outcompete the target for charges on droplet surfaces (Bruins, 1998; Jessome and Volmer, 2006) (Figure 6.1). A number of alternative mechanisms for ion suppression induced by matrix effects have also been proposed and reviewed, mostly specific to the ionization methods employed (Jessome and Volmer, 2006).

Two most widely used methods to evaluate the extent of matrix effects include post-column infusion (Bonfiglio et al., 1999) and post-extraction spike method (Matuszewski, 2006; Matuszewski et al., 2003; Qu et al., 2001). The post-column infusion method assesses matrix effects by continuously infusing a target analyte into the post-column LC flow, which delivers a blank biological matrix sample (Bakhtiar and Majumdar, 2007). This method enables qualitative identification of matrix effects but provides little quantitative information (Van Eckhaut et al., 2009). The post-extraction spike method, on the other hand, enables more quantitative evaluation of matrix effects. The signal response of a known level of target analyte spiked into an extracted blank biological matrix is compared with that from the same amount of spiked-in analyte in neat solutions (Chambers et al., 2007; Lv, 2010; Matuszewski et al., 2003). Alternatively, the experimentally acquired increase of analyte amount before versus after spiking, converted from signal responses, can be contrasted against the actual increase of amount (Qu et al., 2001). The disparity between the responses is defined as the absolute matrix effects for the biological matrix evaluated. Alternatively, the disparity

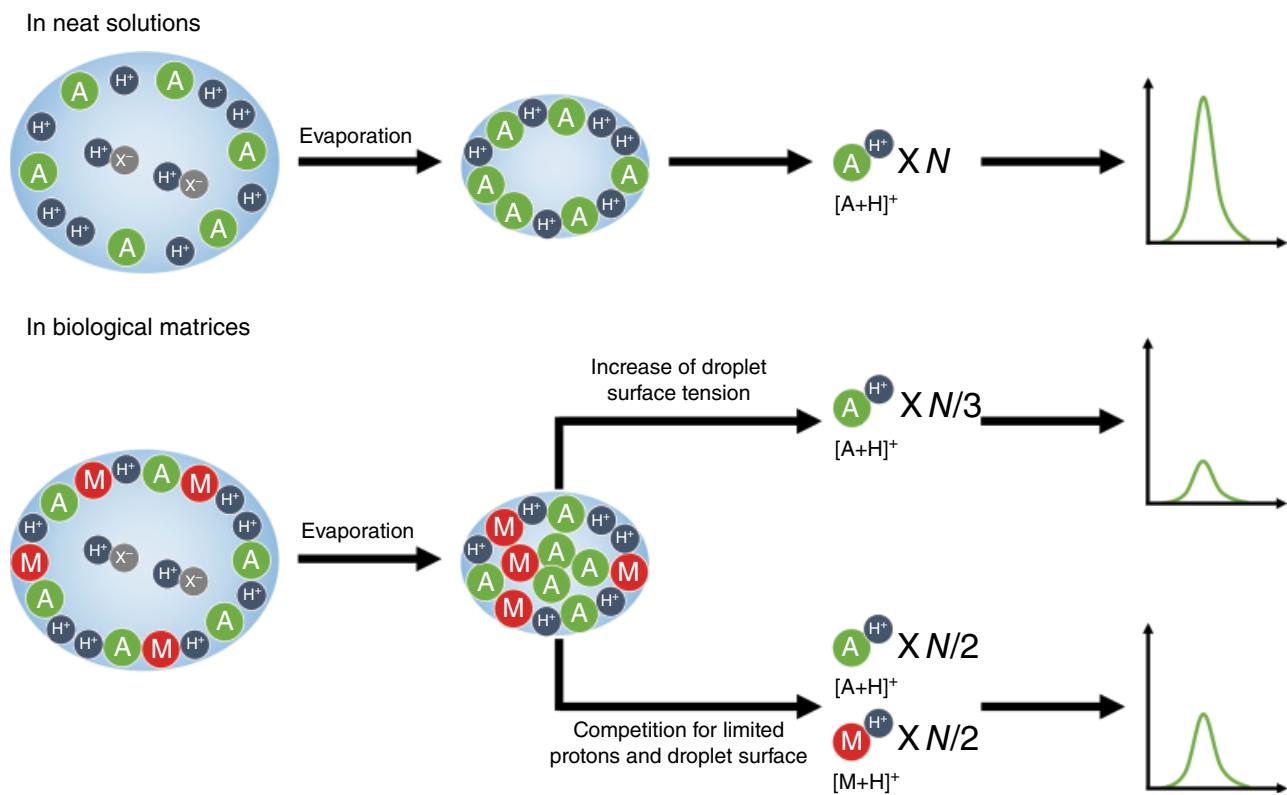


Figure 6.1 Putative mechanisms for matrix effects in ESI.

between the responses normalized to internal standards (ISs) is defined as the normalized matrix effects. More importantly, relative matrix effects could be determined by comparing different absolute matrix effect values among a variety of biological matrices. Matuszewski et al. further suggested that the precision of the slopes from calibration curves in five lots of biological matrices, rather than in a single lot analyzed repeatedly (e.g.,  $n = 5$ ), should be used to better evaluate relative matrix effects. The precision value, defined as the variability of curve slopes from different lots of biomatrices, should not exceed 3–4%. Otherwise, the method could be susceptible to matrix effects as the authors suggest (Matuszewski, 2006).

Elimination or at least alleviation of matrix effects has become necessary during the development of the analytical workflow for LC-MS-based biomarker quantification, which has been comprehensively reviewed (Van Eeckhaut et al., 2009). Briefly, elimination or alleviation of matrix effects could be conducted through effective sample preparation, proper chromatographic, and mass spectrometric conditions, as well as the use of appropriate ISs (Hernandez et al., 2005; Niessen et al., 2006). It is also a common practice to combine several approaches to eliminate matrix effects (Niessen et al., 2006). From the perspective of sample preparation techniques, matrix effects are the immediate aftermath of insufficient

removal of interfering substances, hence ensuring adequate sample cleanup is of profound importance for mitigating matrix effects. A more detailed discussion about the performance of different sample preparation strategies on eliminating matrix effects is elaborated in the following sections.

#### 6.2.1.2 Sensitivity and Selectivity

Sensitivity and selectivity are two major considerations for bioanalysis. While quantification of small molecules by LC-MS is often considered to be sensitive, insufficient sensitivity remains a problem in many cases due to low concentration of target molecules in the biological matrix, or poor responses of target molecules in MS, etc. Though LC-MS is considered to carry excellent selectivity, issues such as interferences from isomeric compounds or chemical noises should be properly addressed (Duan et al., 2010), because such problems could otherwise hamper the detection of target molecules. Therefore, application of optimal sample preparation strategies is critically important for sensitive and selective LC-MS analysis. In general, selective sample preparation procedures such as LLE, SPE, and their derivative techniques can often improve sensitivity and selectivity of the entire analytical procedure, while nonselective methods such as dilute-and-shoot and PPT are not so effective

(Table 6.1). Performances of these techniques on improving sensitivity and selectivity are expounded in Sections 6.2.2.3 and 6.2.2.4.

#### 6.2.1.3 Selection of Calibration Methods

Whenever feasible, the use of proper IS, such as stable isotope-labeled (SIL) IS, is important to alleviate the impacts of matrix effects on sample preparation and LC-MS analysis. The use of deuterium-labeled IS should be avoided if <sup>13</sup>C-, <sup>15</sup>N-, and <sup>18</sup>O-labeled counterparts are commercially available (Wang et al., 2007). Different extents of ion suppression effects and chromatographic behaviors have been observed between the target analyte and deuterium-labeled IS, which may compromise quantitative accuracy and precision (Briscoe et al., 2007; Jemal et al., 2003). Moreover, in some cases deuterium-labeled IS may undergo deuterium–hydrogen back-exchange, potentially leading to positive biases of the results (Davies et al., 2010). When the choice for SIL-IS is limited to deuterium-labeled ones, it is necessary to thoroughly investigate whether the aforementioned aspects (i.e., matrix effects and stability) will affect the quantitative results in the particular study. Otherwise, non-SIL-IS can also be used as an option, yet the quantitative method should be extensively evaluated and validated for biases and variations. Finally, chemical behaviors of the target analyte and IS should also be considered when using IS. For small molecules, the affinity of the analyte toward proteins should be examined and compared with that of the IS selected, as was first demonstrated by Taylor et al. for corticosteroids (Taylor and Harker, 2006). After spiking SIL-IS into the matrix, a certain amount of time should be allowed for the IS to bind proteins until reaching an equilibrium state (Mulvana, 2010). Whether the releasing step (e.g., adding organic solvents, reducing agents) causes same effects on the analyte and IS should be inspected, especially when non-SIL-IS is used (Bylda et al., 2014).

### 6.2.2 Sample Preparation Techniques

#### 6.2.2.1 Dilute-and-Shoot

Dilute-and-shoot is the most straightforward sample preparation approach. Very little or no sample cleanup or target enrichment is employed with this strategy, and thus this method is usually easy to develop, high throughput at the cost of being nonselective (Henion et al., 1998) with no improvement on sensitivity either. This strategy does work when the target biomarker has a high concentration in the sample and the sample matrix is considerably simple (i.e., the impact of matrix effects is negligible, e.g., pharmaceutical preparation or urine samples). For biological matrices with more complex composition, the use of dilute-and-shoot for sample preparation is often not practical.

#### 6.2.2.2 Protein Precipitation (PPT)

Compared with dilute-and-shoot, PPT removes protein contents in the sample matrix and usually facilitates to release the target biomarker sequestered by protein binding. Proteins are usually denatured by organic solvents such as acetonitrile and methanol (Englard and Seifter, 1990), and denatured proteins are pelleted and removed by centrifugation, while the supernatant portion containing the target small molecule is transferred for quantification. PPT enables simple and fast sample preparation yet is also a nonselective method since most small molecule interferences eliciting matrix effects remain.

More recently, improved PPT methods such as PPT plates have been developed to improve the efficiency and throughput. First reported by Biddlecombe and Pleasance (1999) and Williams et al. (2003), PPT plates achieve protein removal by vacuum or centrifugation filtration after precipitation, allowing more automated sample preparation by eliminating the supernatant transfer steps (Kocan et al., 2006). Results from studies comparing PPT plates with conventional PPT methods suggested significant improvement in accuracy, precision, and sample preparation time (Walter et al., 2001). Other advantages include higher sampling throughput, better supernatant recovery, applicability with small amount of samples, and better sample cleanup. PPT plates are now available from a number of vendors in various volumes, membrane/depth filters, and other individual features. A number of parameters including organic solvents used, filter material and pore size, and nonspecific binding should be taken into account when selecting a PPT plate to use (Kole et al., 2011).

#### 6.2.2.3 Liquid–Liquid Extraction (LLE)

LLE, also known as solvent extraction, is one of the most popular sample preparation methods for small molecule biomarkers. LLE utilizes the differential solubilities of compounds in two immiscible liquids to extract target analytes and deplete interfering substances. Usually, the solvent system consists of one hydrophilic solvent (e.g., water, alcohols) and one lipophilic solvent (e.g., chloroform, dichloromethane) (Ni et al., 2008; Oiestad et al., 2009; Tracqui et al., 1997) that are immiscible. Ideally, the target analyte should be enriched in one solvent (i.e., extract) after extraction, while the interfering substances remain in the other solvent (i.e., raffinate). Therefore, the determination of an optimal solvent/buffer system is the key for successful analyte enrichment and interference depletion. Apart from the choice of solvents, sample pH and extraction time are two parameters that need to be evaluated to obtain optimal outcomes. It is suggested that sample pH be tuned to render the least charge of

the analytes, since better extraction performance with maximal selectivity is most likely to be obtained when the analyte is non-charged (i.e., least polar) (Hendriks et al., 2007); in terms of extraction time, it depends on the nature of the analyte and matrix and is not necessarily to be long. It has been demonstrated that 5 min extraction outperformed 20 min for certain tested compounds in terms of interference depletion and analyte recovery, potentially implying that interfering substances may diffuse into the extract if the extraction time was too long (Aubry, 2011).

Conventional LLE procedures often suffer from multiple disadvantages, including inferior selectivity compared with SPE, low or variable analyte recovery due to repeated extractions and emulsions, low efficiency for extracting highly hydrophilic analytes, and difficulties in automated operation (Capka and Carter, 2007; Novakova and Vlckova, 2009; Zhou et al., 2005). To address these disadvantages, several modified LLE methods have been developed and applied to the preparation of biological samples for LC-MS analysis. Some of these are exemplified later.

**6.2.2.3.1 Salt-Assisted Liquid-Liquid Extraction (SALLE)** In SALLE, a high concentration of inorganic salt is added to a solvent system consisting of water and a water-miscible organic solvent, which adds to the polarity of the water phase and leads to phase separation. This phenomenon is termed as “salt-induced phase separation” (Frankforter and Cohen, 1914). A variety of salts with distinct physicochemical properties (e.g., structure, molecular weight (MW), and volatility) are suitable for SALLE, such as magnesium sulfate (Zhang et al., 2009), ammonium sulfate (Rustum, 1989), sodium chloride (Yoshida et al., 2004), calcium sulfate, calcium chloride, and potassium carbonate (Buratti et al., 2006). SALLE was first used in the analysis of trace elements by Matkovich and Christian (1973) and later became quite popular in environmental and food analysis, for example, tetracyclines in surface water and milk (Tsai et al., 2010) and methylene blue and sunset yellow in wastewater and food (Razmara et al., 2011). There are also wide applications of SALLE in the bioanalysis, for example, ABT-869 and its metabolite A-849529 in human blood plasma (Wu et al., 2008),  $\gamma$ -hydroxybutyrate and its precursor metabolites in blood and urine samples (Kankaanpaa et al., 2007), mycotoxin (including highly polar compounds such as deoxynivalenol and fumonisin B<sub>1</sub>) in pig urine (Song et al., 2013), and alkyl methylphosphonic acid in human serum and urine (Roen et al., 2014). Recently, the method developed allowed fast detection of nerve agent exposure in less than 30 min, which was critical for medical countermeasures (Roen et al., 2014). Overall, SALLE surpasses conventional LLE

by offering better extraction efficiency for molecules with high hydrophilicity, as well as improved and more reproducible analyte recoveries, adding to the sensitivity and selectivity of LC-MS analysis. A variation of SALLE employing the similar concept is solvent-induced phase transition extraction (SIPTE) (Liu et al., 2010). By adding a series of more hydrophobic solvents to a water-acetonitrile system, a biphasic system could be established and was used for the extraction of analytes from biological samples.

**6.2.2.3.2 Solid-Supported Liquid Extraction (SLE)** Another modified LLE method is solid-supported liquid extraction (SLE), which aims at solving the low sample throughput and poor automation problem for conventional LLE method. In SLE, samples containing the target analyte are first loaded onto a porous solid support material (e.g., diatomaceous earth (Bylda et al., 2014), polypropylene hollow fiber (Busquets et al., 2009)) in a plate or cartridge, and a quick vacuum pulse, and a certain length of time is allowed for the sample to be maximally absorbed by the material (unlike SPE, there is no flow-through in the loading step). The other solvent is then flowed through the material for several times, which elutes the target analyte while the interfering substances stay absorbed in the solid support material. Compared with conventional LLE, SLE achieves better analyte recovery (Zhang et al., 2013a) and excellent phospholipid removal when conditions are well optimized (Ismaiel et al., 2010), meanwhile significantly increasing the analytical throughput of sample preparation (Singleton, 2012). Former studies have shown a highest 99.5% removal rate of phospholipids when dichloromethane was used, followed by 99% using methyl-*tert*-butyl ether and 85% using ethyl acetate (Jiang et al., 2012). Water-miscible solvents such as acetonitrile and methanol are not recommended for SLE, since they will compromise phospholipid removal, leading to elevated matrix effects (Jiang et al., 2012). Using SLE, the sample preparation procedures could be automated with much higher throughput. For example, Wu et al. compared the analytical performance of a 96-well SLE method with conventional LLE method to quantify hydrocortisone in mouse serum. With similar sample cleanup and analyte recovery, SLE shortened the sample preparation time by approximately 50 min, enabling high-throughput sample preparation in approximately 35 min (Wu et al., 2010). SLE has extensive applications in the bioanalysis of drug compounds and their metabolites. To list a few, SLE has been employed for the quantitative analysis of PD 0332991 in xenograft mouse tumor tissue (Nguyen et al., 2010), molindone enantiomers in human plasma (Jiang et al., 2008), and erlotinib in human plasma (Pan et al., 2010). All of these

studies achieved outstanding quantitative accuracy and precision with the implementation of SLE technique.

Apart from SALLE and SLE, a few other liquid extraction methods based on LLE have also been devised and utilized for sample preparation for LC-MS analysis, for example, EXtrelut LLE, liquid–liquid microextraction (LLME), and pressurized liquid extraction (PLE). The principles and applications of these methods are elaborated in a previous review by Nováková and Vlckova (2009).

#### **6.2.2.4 Solid-Phase Extraction (SPE)**

SPE is another ubiquitously used sample preparation strategy. Using principles similar with LC, SPE partitions target analyte from interfering substances in the sample matrix by their differences in retention behaviors on the stationary phase (Berrueta et al., 1995). After sample loading, washing, and mobile phase elution, the target analyte could be eluted into the flow-through, while interfering substances remain on the stationary phase, and vice versa. Generally, SPE can be categorized into three modes based on their mechanisms for separation: normal-phase, reverse-phase, and ion exchange modes (Hennion, 1999). “Multimodal SPE,” such as mixed-mode cation exchange (MCX) SPE, has also been developed by incorporating multiple retention mechanisms in one SPE procedure (Koole et al., 1999). This allows more selective and specific extraction of target analytes than LLE, endowing a more versatile role of SPE in sample preparation for analytes with various physicochemical properties. The method development process of SPE is similar with that for LC, in which one key factor contributing to the final outcomes is the selection of stationary phases. A wide range of SPE sorbents are commercially available, including chemically bonded silica of C8 and C18 groups, graphitized carbon, ion exchange materials, polymeric materials, mixed-mode sorbents, immuno-sorbents, molecularly imprinted polymers, and RAM. The pros and cons of these SPE sorbents are described thoroughly (Novakova and Vlckova, 2009). Several important points are exemplified here. Polymer sorbents are more tolerant than silica ones to acidic and basic conditions and also suffer less from irreversible binding for certain compounds, for example, tetracyclines (Fontanals et al., 2005; Hennion, 1999). Besides excellent recovery for target analyte and significant depletion of matrix interferences, SPE offers several additional advantages over LLE, including easy preconcentration of analytes, little emulsion formation, and better automated operation (Kataoka, 2003). Nonetheless, SPE also bears minor issues such as relatively higher expenses and occasionally poor inter-batch reproducibility (Novakova and Vlckova, 2009). Another potential caveat of using conventional SPE comes from the SPE cartridges/plates,

in which endogenous impurities of SPE material (e.g., polyethylene glycols and phthalates) may be co-eluted with the analyte by the mobile phase and trigger interferences for the LC-MS analysis. This issue, which may compromise the sample cleanup capability of SPE, has been described (Aurand, 2006). To resolve this problem and meanwhile provide better selectivity, various modified SPE methods and SPE-based methods have been designed and tested, as archived in two reviews (Kole et al., 2011; Novakova and Vlckova, 2009). Notably, online SPE (OLSPE) has significantly enhanced analytical throughput and robustness of bioanalysis. OLSPE brings an SPE column in tandem with the LC-MS system, thus alleviating inter-analyst variability and shortening the time needed for sample preparation (Singleton, 2012). Various sorbents can be used for OLSPE; meanwhile commercial OLSPE columns are also mainly available in two forms: RAM columns and turbulent-flow chromatography (TFC) columns (Kole et al., 2011).

#### **6.2.2.4.1 SPE-PPT**

The combination of SPE and PPT is employed to facilitate the extraction of highly hydrophobic compounds with high affinity to proteins. As reported previously, this combination enabled elevated analyte recovery to nearly complete (Lindegardh and Bergqvist, 2000). One representative commercial product available on market is HybridSPE-PPT<sup>TM</sup> by Sigma-Aldrich. HybridSPE-PPT procedure encompasses two major phases: First, acetonitrile plus centrifugation is utilized to precipitate protein contents in the samples, and the supernatant portion is loaded onto the HybridSPE part. Second, HybridSPE containing a proprietary zirconia-coated particle is used to specifically remove phospholipids from the samples. HybridSPE-PPT thereby achieves selective elimination of phospholipids along with depletion of various acidic, basic, and neutral compounds (Aurand and Trinh, 2009; Lu and Ye, 2010). Both cartridges and 96-well forms of HybridSPE-PPT have been applied to a number of in vivo quantitative analysis of drug compounds, for example, sitagliptin (Zeng et al., 2010), carboplatin (Jiang et al., 2011), clopidogrel (Silvestro et al., 2010), PDE5 inhibitors (Unceta et al., 2012), and multiple antipsychotic drugs (Sampedro et al., 2012), as well as small molecule biomarkers, for example, amine neurotransmitters (Moriarty et al., 2012), alkylphenols (Asimakopoulos and Thomaidis, 2015), and steroids (Rousu and Tolonen, 2012).

#### **6.2.2.4.2 Solid-Phase Microextraction (SPME)**

Solid-phase microextraction (SPME) is an SPE technique to handle small amount of samples, first developed by Arthur and Pawliszyn in 1990 and then introduced for fast and effective sample preparation in early 1990s (Arthur and Pawliszyn, 1990; Belardi and Pawliszyn, 1989). SPME is a

syringe-based extraction method, which modifies a common syringe by inserting a stainless steel microtubing into the syringe needle (Augusto and Valente, 2002). At the front end of the microtubing, there is a 1 cm fused silica fiber tip that is coated with organic polymer, and the coated fiber could extend or retract with the movement of a plunger. Two common types of SPME are direct immersion SPME (DI-SPME) (Colon and Dimandja, 2004) and headspace SPME (HS-SPME) (Zhang et al., 1994). DI-SPME involves immersion of the fiber tip in the sample matrix by which the tip is directly exposed in the matrix. In HS-SPME, the fiber tip is suspended above the sample matrix in a vial, and the matrix is heated to volatilize the analytes. While HS-SPME better prevents contamination of the fiber tip surface, it also imposes the requirements of high volatility of the analyte to be extracted. SPME accomplishes solvent-free sample preparation in a simple two-step scheme (i.e., extraction and desorption), and both steps should be meticulously optimized according to the matrix and analytes (e.g., pH, salt concentration, sample volume, temperature, desorption solution) (Kole et al., 2011). For the SPME apparatus itself, the physicochemical properties and the thickness of the coating polymer are the two key factors for successful SPME. A number of frequently used polymers include polydimethylsiloxane (PDMS), polyacrylate (PA), polydimethylsiloxane–divinylbenzene (PDMS-DVB), carboxen–polydimethylsiloxane (CAR-PDMS), carbowax–divinylbenzene (CW-DVB), carbowax-templated resin (CW-TPR), and divinylbenzene–carboxen–polydimethylsiloxane (DVB-CAR-PDMS) (Theodoridis et al., 2000). Overall, SPME possesses several advantages including simplicity in use, easiness of automation, minimal consumption of organic solvents, and low requirements for experimental apparatus. Another unique advantage for using SPME for sample preparation is the opportunity to perform on-fiber analyte derivatization simultaneously with analyte extraction by fixing derivatizing agents in the coating polymers (Stashenko and Martínez, 2004). The application of SPME in sample preparation expands widely from numerous drug compounds (e.g., antidepressant drugs (Alves et al., 2007), citalopram (Nezhadali et al., 2012), amphetamine (Myung et al., 1998), barbiturate (Hall and Brodbelt, 1997), halogenated anesthetics (Indelicato et al., 2014)), small molecule biomarkers (e.g., volatile organic compounds for lung cancer (Poli et al., 2005), organic acids in urine (Li et al., 2007), and fatty acids in feces (Zheng et al., 2013)) to toxic substances (e.g., metaldehyde (Saito et al., 2008)). Later, an alternative in-tube SPME besides the traditional fiber SPME has been introduced (Eisert and Pawliszyn, 1997).

**6.2.2.4.3 Stir Bar Sorptive Extraction (SBSE)** Stir bar sorptive extraction (SBSE), emerged in the year of 1999, is a new solvent-less SPE-based technique (Baltussen et al., 1999). SBSE shares the similar principles with SPME yet differs with SPME in that the fiber tip used for polymer coating is replaced by a magnetic stir bar. This altered setting results in a drastic increase of volume for the extraction phase (50–250 times larger) (David and Sandra, 2007), which enables higher extraction efficiency for SBSE in a single extraction procedure (Alves et al., 2005). The selection of organic polymers as the extraction phase, however, is more limited for SBSE, with PDMS being the only material commercially available (Novakova and Vlckova, 2009). Therefore, traditional SBSE is more favored for the extraction of hydrophobic analytes. This limited usage of SBSE has been ameliorated by the utilization of *in situ* derivatization to modify analyte polarity and the development of novel extraction phases for SBSE, for example, PDME/carbon dual-phase (Bicchi et al., 2003). Like SPME, SBSE also has two modes, namely, DI-SBSE and HS-SBSE (David and Sandra, 2007). Sample preparation using SBSE also involves the “extraction-and-desorption” scheme. Each procedure during SBSE also has to be well optimized, with several critical parameters being sample pH value, ratio between sample and extraction phase, speed of stirring, temperature, extraction duration, and salt concentration (Bicchi et al., 2003). Compared with SPME, the desorption process for SBSE is comparatively slower because of the much larger extraction phase used (Pavlović et al., 2007). While thermal desorption and liquid desorption are usually performed for desorption, cold trapping and reconcentration are also recommended to be combined with desorption (Pavlović et al., 2007). One prominent drawback of SBSE is its long extraction duration, which could take up to 150 min to accomplish (David and Sandra, 2007). SBSE has been a popular sample preparation strategy for the analysis of environmental and biomedical samples, as reviewed previously (David and Sandra, 2007; Kawaguchi et al., 2006). SBSE also merges as a promising analyte extraction method for a variety of analytes, for example, steroid hormones (Almeida and Nogueira, 2006), benzophenones (Kawaguchi et al., 2008), antidepressants (Chaves et al., 2007), fluoxetine (Fernandes et al., 2008), and tricosan (Silva and Nogueira, 2008), and in a number of biological matrices, for example, urine and saliva (bisphenol A (Kawaguchi et al., 2004)), plasma (caffeine metabolites (Lambert et al., 2005)), and brain (serotonin uptake inhibitors (Unceta et al., 2010)).

**6.2.2.4.4 Microextraction by Packed Sorbent (MEPS)** Microextraction by packed sorbent (MEPS) is another syringe-based SPE method developed by Abdel-Rehim

et al. at AstraZeneca (Abdel-Rehim, 2004), which could be regarded as a miniature form of SPE. MEPS starts with the packing of 1–2 mg of solid sorbents (e.g., C2, C8, C18, and strong cation exchange (SCX)) into either the barrel of a syringe (100–250 µL) as a plug with polyethylene filters on both ends or between the barrel and needle as a cartridge. The extraction steps of MEPS turn out to be similar with those of conventional SPE. The sample is first aspirated and dispensed for several times with the packed syringe manually or using an autosampler, allowing the binding of analytes to the sorbent. After washing the sorbent with water (~50 µL), the target analyte will be eluted using an appropriate volume of solvent (e.g., 20–50 µL), and the eluent could be injected directly into the LC-MS system. Using MEPS, the sample preparation procedures could be completely automated and connected online to the LC-MS system (Abdel-Rehim, 2010). MEPS also leads to comparably efficient removal of phospholipid as SPE, which significantly promotes the selectivity of LC-MS analysis. Another advantage of MEPS in comparison with SPME is the reusability of the packed syringe. According to one study, MEPS can be reused >100 times for plasma or urine samples and >400 times for water samples (Altun et al., 2004). However, other studies also noticed that matrix components in plasma samples might alter the surface chemistry and sorption properties of the sorbents packed, leading to declined reusability of MEPS (Altun and Abdel-Rehim, 2008). It would be advisable to validate the performance of used MEPS in specific projects before conducting another preparation. A few other drawbacks of MEPS include potential bubble formation and incapability of online connection with certain LC-MS systems. However, capable of handling both small (~10 µL) and large (1000 µL) volume samples and more independent sample matrix (compared with SPME), MEPS has been widely applied in the sample preparation from many types of biological matrices, for example, plasma, serum, urine, whole blood, hair, and saliva. MEPS has been employed for the extraction of drug compounds such as remifentanil (Said et al., 2011), AZD3409 (Abdel-Rehim et al., 2008), ropivacaine and its metabolites (Abdel-Rehim et al., 2004), roscovitine (Vita et al., 2005), atorvastatin (Vlčková et al., 2011), and cyclophosphamide (Said et al., 2008).

A few other SPE-based techniques, for example, monolithic SPE discs/spin columns, molecularly imprinted polymer SPE (MISPE), dispersive solid-phase extraction (dSPE or QuEChERS), disposable pipette extraction (DPX), and TFC, have also gained popularity upon introduction. Details about these techniques have been described thoroughly (Kole et al., 2011).

## 6.3 Sample Preparation Strategies for Macromolecule Biomarkers

Though a wide range of macromolecules can serve as biomarkers, most biomarkers in this category are proteins or long peptides. These biomarkers can present either in circulation, biofluids, or tissues. Sample preparation for macromolecules, compared with that for small molecules, is profoundly more complicated, which requires multiple experimental procedures to accomplish, for example, extraction, enrichment, denaturation, chemical modification, and proteolytic digestion. A schematic illustration of the sample preparation workflow for macromolecule biomarkers is shown in Figure 6.2. Principles, pros and cons, and applications of the procedures involved are covered in this section.

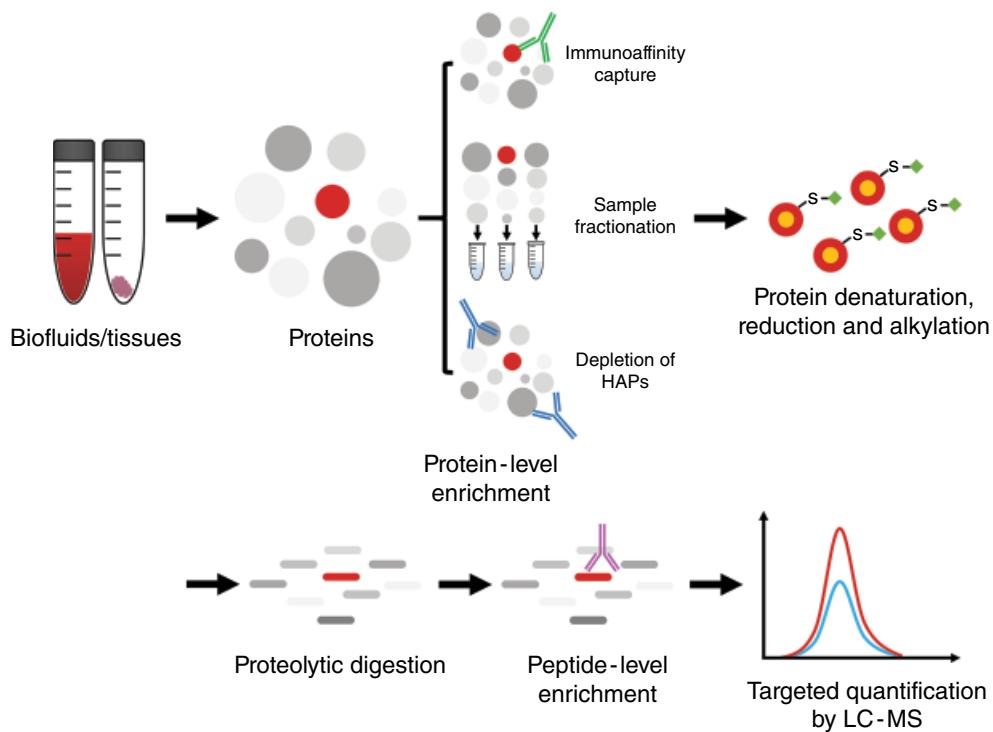
### 6.3.1 Considerations for Sample Preparation

#### 6.3.1.1 Matrix Effects

For targeted quantification of macromolecule biomarkers in biological matrices, matrix effects should also be meticulously evaluated and addressed as expounded in Section 6.2.1.1. Furthermore, the highly complex peptide mixture derived from matrix proteins post digestion could further compromise the quantitative sensitivity and selectivity, which should be addressed.

#### 6.3.1.2 Recovery of the Signature Peptide from the Target Analyte

For targeted quantification of protein biomarkers, it is important to establish optimal protocols for sample cleanup and proteolytic digestion of the target protein that achieve high and reproducible recovery of the SPs. LC-MS-based protein quantification, mostly employing selected reaction monitoring (SRM), is based on the detection of signals from SPs released from the native proteins (Lange et al., 2008). Complete and consistent cleavage of the proteins into proteolytic peptides species is desirable so that the absolute concentration of a protein could be referred by those of the SPs quantified. In reality, however, 100% cleavage is difficult to achieve (Barnidge et al., 2004; Klanner and MacCoss, 2006). Proteins often contain structural features that will thwart enzymes from accessing proteolytic cleavage sites, for example, disulfide bonds, specific folds, hydrophobic domains, and posttranslational modifications (PTMs) (Proc et al., 2010). Some proteins are naturally more resistant to proteolytic enzymes, which may also confer difficulties for efficient digestion (Proc et al., 2010). Therefore, instead of achieving near-complete digestion, whether a reproducible digestion can be achieved and a constant percentage of target SP could be released when



**Figure 6.2** Schematic workflow of sample preparation of targeted quantification of macromolecule biomarkers.

digestion finishes is of higher priority for quantification work (Hervey et al., 2007; Klammer and MacCoss, 2006; McDonald et al., 2002; Russell et al., 2001). As proposed by Proc et al., an ideal digestion protocol should accomplish digestion in a relatively short period of time, achieve maximal digestion efficiency and reproducibility upon completion, and could be maintained at a “steady state” during which no significant changes in peptide concentration are observed (Proc et al., 2010). To achieve efficient, reproducible, and robust proteolytic digestion, rigorous evaluation and optimization should be conducted for every step within the digestion workflow. More specifically, several key conditions of the digestion process ought to be optimized upon the digestion methods selected (expounded in Section 6.3.5).

The first element to consider is which enzyme and what substrate-to-enzyme (S:E) ratio to use for digestion. The predominantly used proteolytic enzyme for both quantitative proteomics and targeted quantification is trypsin, a serine protease cleaving peptide bonds at the carboxyl side of lysine and arginine except when either is bound to a C-terminal proline. Since lysine and arginine residues are relatively abundant and well distributed in the majority of protein molecules, the use of trypsin enables highly specific generation of proteolytic peptides with feasible lengths (Brownridge and Beynon, 2011; Vandermarliere et al., 2013). A large proportion of tryptic peptide ions exists in  $[M+2H]^{2+}$  under ESI

ionization, a form that is preferable for tandem MS fragmentation (Brownridge and Beynon, 2011; Burkhart et al., 2012). Moreover, trypsin outcompetes its counterparts by its decent affordability (Dittrich et al., 2015). Trypsin produced from distinct origins and manufacturers may differ in proteolytic activity. For example, one study has demonstrated that the usage of bovine-origin trypsin led to more miscleavages, while a higher percentage of semi-tryptic peptides were generated by porcine-origin trypsin (Burkhart et al., 2012). In certain cases, peptides generated by trypsin may not be suitable for targeted quantification. Under these circumstances, alternative proteolytic enzymes with different cleavage patterns may be an option for protein digestion, for example, Lys-C, Asp-N, Glu-C, Arg-C, and Asp-N. As to the S:E ratio, generally, an S:E ratio between 1:10 and 1:20 (w/w) works well for most proteins, as corroborated by Hustoft et al. (2011). Yet, it is recommended to perform an optimization step for individual proteins to be cleaved, especially those proteins with multiple protease-resistant domains.

Composition of the digestion matrix also contributes significantly to digestion and peptide recovery. Prior to digestion, solubilized proteins usually undergo pretreatments including denaturation, reduction, and alkylation, with optional protein/peptide-level enrichment (elaborated in Section 6.3.3) and cleanup. These procedures largely determine the complexity of the digestion

solution matrix, which correlates with digestion rate and efficiency. General conditions that need to be adjusted before digestion include protein concentration, pH, and salt concentration. Chaotropic agents should be depleted via desalting or at least diluted to a low concentration (e.g., <1 M) to minimize their denaturing power upon enzymes and to circumvent the nonspecific modification of certain amino acid residues (Kraus et al., 1994; Stark et al., 1960). Surfactants, such as sodium dodecyl sulfate (SDS), Triton X-100, and NP-40, should also be removed from the matrix, as they could also denature proteolytic enzymes and cause ion suppression effects on ESI (Antharavally et al., 2011). The use of MS-compatible surfactants could address this issue to a certain extent. Several experimental procedures to remove surfactants encompass MW-based cutoff filters (as in filter-aided sample preparation (FASP)) (Wisniewski et al., 2009), solvent partition/precipitation of proteins (e.g., acetone, chloroform/methanol, ethyl acetate) (An et al., 2015; Duan et al., 2009; Vertommen et al., 2010; Yeung and Stanley, 2010), and KCl precipitation of SDS (Carraro et al., 1994). Moreover, matrix proteins, especially those in a complex biomatrix such as plasma, can potentially influence proteolytic digestion efficiency. Therefore, protein enrichment (e.g., immunoaffinity capture (IC)), capable of alleviating interferences from those matrix proteins, may also facilitate the digestion process.

Another consideration for optimization of the digestion process is the temperature and length of the enzymatic reaction. While the optimal working temperature for trypsin is 37°C, several previous reports suggested that increasing the reaction temperature to 50–60°C via direct heating or microwave could remarkably expedite proteolytic cleavage (Zhang et al., 2013b). However, heat-induced denaturation of native trypsin may also happen under high temperatures and lead to compromised digestion efficiency, as is supported by various studies (Finehout et al., 2005). Chemical modifications of native trypsin add to its thermostability and allow a 50°C reaction temperature; however only a marginal decrease of digestion time was observed, as shown by Loziuk et al. (2013). Another approach to increase trypsin thermostability is the immobilization of trypsin on a support material, which prevents trypsin autolysis under high temperatures (Freije et al., 2005; Qin et al., 2012). Immobilized trypsin is available in various forms, including nanoparticles (Kim et al., 2010; Qin et al., 2012), columns (Calleri et al., 2004; Slysz and Schriemer, 2003), pipette tips (Hahn et al., 2009; Ota et al., 2007), and microchips (Bao et al., 2013; Fan et al., 2013), which could be reused and automated easily. The applicability of immobilized trypsin in targeted quantification studies still warrants further investigation. The length of the digestion process should also be monitored and modified

for the proteins to be quantified, preferably in pilot studies, since, for different proteins and SPs, the digestion length required may vary drastically. For instance, Ceglarek et al. have shown that the time demanded for the formation of two SPs from apolipoprotein A-I and A-II, respectively, could differ by >15 h (Ceglarek et al., 2013). An ideal digestion length should be identified to allow complete formation of target SPs. When multiple proteins are to be digested and analyzed together, a comprehensive assessment of digestion efficiency under different lengths should be performed, as is exemplified by Proc et al. (2010).

#### 6.3.1.3 Selection of Calibration Methods

The selection of calibration methods may profoundly affect the accuracy and precision of protein analysis. Potential sources of variation and bias include but not limited to protein loss during sample treatment, suboptimal peptide recovery due to issues such as missed cleavages and peptide degradation (Fernandez Ocana et al., 2012), and the improper use of calibration methods (Nouri-Nigjeh et al., 2014). Using full-length SIL protein IS is thus desirable because the IS will undergo the same sample preparation and proteolytic cleavage processes as the analyte does (Heudi et al., 2008; Kuhn et al., 2012; Li et al., 2012). However, production of full-length SIL-IS could be extremely cost prohibitive since high isotope purity is required, and this becomes almost impractical when multiple protein ISs are needed simultaneously (van den Broek et al., 2013). As cheaper alternatives, peptide-level (Keshishian et al., 2007; Pan et al., 2009) or extended peptide-level (i.e., target peptide with three to six flanking residues extended from both N- and C-termini) (Neubert et al., 2013; Rauh, 2012) SIL-ISs combined with calibrators on different levels (i.e., protein level, extended peptide level, peptide level) have been used as a substitute of the full-length IS. The quantitative performance of different combinations between calibrators and SIL-ISs has been thoroughly assessed by Nouri-Nigjeh et al. (2014), and according to the results, a “hybrid” calibration method using full-length protein calibrators with either peptide-level or extended peptide-level SIL-ISs achieved superior quantitative accuracy and precision, as well as better consistency of quantitative data between two SPs selected. This “hybrid” method offers a more cost-effective alternative for calibration, while high quality quantitation could be achieved, provided the sample preparation procedure is highly reproducible (Cao et al., 2010; Nouri-Nigjeh et al., 2014). Similarly, Arnold et al. used the “hybrid” method and obtained high quality quantitative results for microsomal retinol dehydrogenase (RDH11) and cytosolic soluble aldehyde dehydrogenase (ALDH1As) in human liver tissue (Arnold et al., 2016).

#### 6.3.1.4 Sensitivity and Selectivity

Insufficient sensitivity and selectivity are two of the most prominent challenges for LC-MS-based quantification of proteins in biomatrices (Qu et al., 2016). There are two primary factors compromising the sensitivity of LC-MS-based protein quantification. First, as ESI responses are molarity dependent rather than mass concentration dependent (Banks, 1996; Mitulovic et al., 2003), and thus the high MW of proteins, compared with small molecules, poses an intrinsic downside. Second, the high protein concentrations in biomatrices such as plasma (30–80 mg/mL) (Zaias et al., 2009) and tissues (30–200 mg/g) (Zaias et al., 2000) necessitate a sample dilution step often >10-fold prior to sample preparation procedures, which further decreases the concentration of biomarkers to be quantified in the matrix. The additive result of these two factors leads to significant sensitivity issue for LC-MS-based protein quantification. On the other hand, selective quantification of target peptides is plagued by endogenous interferences from peptides released by matrix proteins post digestion. In addition, the need to quantify specific protein forms (e.g., PTMs, cleaved proteins, bound proteins) further complicates the situation (Qu et al., 2016).

Improvement of the sensitivity and selectivity of LC-MS-based protein analysis can be achieved on multiple levels. Efficient production and recovery of SPs via proteolytic digestion, as well as enrichment of target analyte on protein and peptide levels, are two predominant measures to increase assay sensitivity and selectivity. Principles and details of techniques used in these two aspects are discussed in detail in Sections 6.3.3 and 6.3.5.

#### 6.3.2 Methods for Protein Extraction

In LC-MS-based protein biomarker quantification, the initial phase is to have an aqueous sample form with a reasonable concentration of the target protein. Biofluid samples, for example, serum/plasma, urine, and saliva, are cell-free matrices in which proteins have already been solubilized. For tissue samples, however, most protein contents are contained within cells, and thus an additional procedure is required to lyse the cells and release cellular proteins into an aqueous solution/suspension. A routine workflow for protein extraction from tissue samples encompasses three major steps: tissue homogenization and disruption, cell membrane disruption (e.g., mechanical force, sonication, freeze/thaw), and centrifugation to separate soluble from insoluble components (Shevchenko et al., 2012). The protein-rich supernatant after centrifugation is then used for subsequent sample preparation procedures. Commonly used buffer systems for protein extraction include phosphate-buffered saline (PBS), Tris-HCl/FA, ammonium bicarbonate (ABC),

and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Salts (e.g., NaCl, KCl) and metal chelators (e.g., ethylenediaminetetraacetic acid (EDTA)) are also frequently added to extraction buffers to provide necessary ionic strength and metal chelating capacity. To obtain better recovery of cellular proteins, especially those hydrophobic membrane-bound proteins, various surfactants and chaotropic agents have also been supplemented to the extraction buffer. Addition of surfactants to extraction buffers has been proved to significantly elevate protein recovery, especially for hydrophobic membrane-bound proteins, potentially via interaction with the bilipid layers of cellular membrane and organelle membrane and help solubilize hydrophobic proteins (Arachea et al., 2012). The positive and negative aspects of using surfactants and chaotropic agents during sample preparation are elaborated in Section 6.3.4. Another indispensable component of extraction buffer is protease inhibitors (e.g., aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and pepstatin A) that protect extracted proteins from proteolytic cleavage by endogenous proteases. Commercial protease inhibitor cocktail tablets are widely available, such as cComplete<sup>TM</sup> from Roche, SIGMAFAST<sup>TM</sup> from Sigma-Aldrich, and UltraCruz<sup>TM</sup> from Santa Cruz.

#### 6.3.3 Methods for Protein and Peptide Enrichment

In a biological matrix, the dynamic range of protein concentrations can exceed 10 orders of magnitude (Anderson and Anderson, 2002), in which over 90% of the total protein contents is occupied by high abundance proteins (HAPs) (e.g., serum albumin, immunoglobulin, hemoglobins, cytoskeleton constituents, histones) (Dittrich et al., 2015). Hence, quantification of medium abundance protein (MAPs) and HAPs in these matrices could be performed directly in a simple and straightforward scheme (Anderson and Hunter, 2006; Ceglarek et al., 2013). For low abundance proteins (LAPs), however, their low concentrations as well as the high background arising from matrix components, poses tremendous challenges for quantitative analysis. In this context, enrichment approaches are necessary to achieve sensitive, accurate, and precise quantification of low abundance biomarkers. Enrichment of target biomarkers can be conducted before (i.e., protein level) or after proteolytic cleavage (i.e., peptide level), and generally, prevalent approaches for protein/peptide enrichment include IC of target proteins/peptides, fractionation of whole protein samples, and depletion of HAPs. Although distinct in principles and experimental settings, these approaches aim at one goal: to decrease the dynamic range of the biological matrix and to achieve sufficient

quantitative sensitivity. Under certain circumstances, multiple approaches could be applied in tandem to provide more selective and effective enrichment of the target analyte.

#### 6.3.3.1 Immunoaffinity Capture (IC)

In the context of LC-MS-based protein quantification, IC serves to capture target protein/peptide analytes from the original samples using one or multiple antibodies immobilized to a certain support (e.g., Sepharose beads, acrylamide polymers, POROS). A typical IC procedure encompasses two major steps. First, samples are loaded onto the antibody-linked support, and a certain length of time is allowed for target analytes to bind with the corresponding antibody. Irrelevant compounds are then depleted with a washing buffer. Second, captured analytes are eluted by dissociating the antibody–antigen interaction with an elution buffer in a controlled volume (Moser and Hage, 2010). The implementation of IC to the targeted quantification workflow offers several remarkable advantages, such as simplification of sample matrix, selective concentration of target analyte (by up to 1000 fold), decrease of LC-MS chemical noise, and potential facilitation to the digestion process (Ackermann and Berna, 2014). A number of considerations should be taken into account to develop an optimized IC workflow with high efficiency and reproducibility, for example, availability and costs of antibodies, antibody specificity, sample matrix and volume, sample dynamic range, and throughput. IC of proteins is widely used in quantitative proteomics and has been adopted later for targeted protein quantification (Fredolini et al., 2016). IC-LC-MS assays have been devised and used for the quantification of serum biomarkers such as human chorionic gonadotropin (Lund et al., 2012, 2013; Woldemariam and Butch, 2014), progastrin-releasing peptide (Torsetnes et al., 2012, 2014), and neuron-specific enolase (Torsetnes et al., 2013). On the other hand, since most LC-MS-based protein quantification methods rely on the quantification of SPs as surrogates for the target proteins, IC on the peptide level is also regarded as a pertinent option. One preferred method, stable isotope standards and capture by anti-peptide antibodies (SISCAPA) was developed and introduced by Anderson et al. in 2004 (Anderson et al., 2004b). SISCAPA utilizes antibodies cross-linked on solid support (e.g., POROS columns, magnetic beads) to specifically capture SPs and SIL-ISs for LC-MS analysis. Since ISs are spiked into the sample matrix prior to SISCAPA, bias and variability from the capture procedure (e.g., endogenous interferences) are minimized (Fredolini et al., 2016). The enriched SPs and corresponding SIL-ISs are eluted into a less complex and more MS-friendly matrix for downstream analysis. Previous studies have demonstrated that SISCAPA could

significantly enhance the sensitivity of the analysis (Whiteaker et al., 2010, 2011b). Moreover, SISCAPA appears to be quite reproducible, with low intra- and inter-lab coefficient of variation (CV) (Whiteaker et al., 2010, 2011b). A number of studies have demonstrated the use of SISCAPA in quantitative analysis of protein biomarkers, for example, interleukin 33 and cardiac troponin I (Kuhn et al., 2009), thyroglobulin (Hoofnagle et al., 2008), metallopeptidase inhibitor 1 (Ahn et al., 2012), and protein C inhibitor (Razavi et al., 2013). Multiplexed SISCAPA-LC-MS assays have also been developed to enable simultaneous quantification of a large number of proteins, as reported in the literature (Keshishian et al., 2007; Whiteaker et al., 2010, 2011b). In addition, tandem IC of intact proteins and proteolytic peptides has been shown (Neubert et al., 2013). As a proof of concept, serum human  $\beta$ -nerve growth factor was first enriched offline, digested along with extended SIL-IS, and SPs generated were enriched online by SISCAPA for LC-MS analysis. It should also be noted that several limitations of SISCAPA may potentially hamper its application, including low availability and high costs of anti-peptide antibodies, nonspecific binding of interfering matrix peptides, and inefficient recovery for certain SPs (Whiteaker et al., 2010, 2011a).

#### 6.3.3.2 Sample Fractionation

Sample fractionation is another commonly applied approach to reduce high abundance interferences and thus enhance the detection of low abundance analyte. By adjusting the separation gradient elution, the target analytes (i.e., protein or proteolytic peptides) could be enriched into one of the multiple fractions with a less complicated matrix composition. Sample fractionation approaches endow tremendous benefits for improving the sensitivity of LC-MS assay, though sample throughput can be a potential pitfall as individual fractions have to be examined and analyzed separately.

Two primary types of fractionation employed in targeted protein quantification are electrophoresis-based fractionation and chromatography-based fractionation. Electrophoresis-based fractionation, predominantly in the form of polyacrylamide gel electrophoresis (PAGE), accomplishes protein separation by its molecular mass (e.g., SDS-PAGE) (Kirkpatrick et al., 2005). Separation of proteins by their isoelectric points (i.e., isoelectric focusing (IEF)) is an alternative approach (Shi et al., 2012b). After staining by Coomassie Brilliant Blue, gel bands containing the target proteins are excised and undergo in-gel digestion (*vide infra*) to release SPs for analysis. A more advanced gel fractionation method is two-dimensional gel electrophoresis (2-DE), which combines the separating power of SDS-PAGE and IEF (Rabilloud et al., 2010). By sequentially applying a pH gradient and

an electrical potential in an orthogonal orientation, 2-DE could achieve high-resolution separation of thousands of proteins on a single polyacrylamide gel. Gel fractionation coupled to LC-MS analysis was applied in the quantification of biomarkers, such as colorectal cancer biomarkers in feces (Ang and Nice, 2010), ovarian cancer biomarkers in serum (Elschenbroich et al., 2011), cytokines, chemokines, and growth factors in plasma (Ahn and Khan, 2014). Besides gel electrophoresis, free-flow electrophoresis, an analogous technique to capillary electrophoresis, has also been practiced for sample fractionation (Nissum and Foucher, 2008).

Chromatography is the other category of fractionation approaches utilized for enriching target analytes, which could be performed on both protein and peptide levels. While theoretically most types of chromatography are applicable for this purpose, techniques that have high separation power, good availability, and mechanisms orthogonal to conventional reversed-phase chromatography (RPLC)-MS used for peptide quantification, are preferred. Among these, SCX chromatography turns out to be the most popular choice (Shi et al., 2012b). Coupling offline or online SCX fractionation with LC-MS analysis for better quantitative sensitivity is a common practice adopted by a number of studies quantifying putative clinical biomarkers in biological matrices, as demonstrated by a number of biomarker quantification works (DeSouza et al., 2008, 2009, 2010; Shah et al., 2009). Recently, high-pH reversed-phase chromatography (RPLC) emerges as a powerful alternative for SCX. Shi et al. showed that compared with SCX, high-pH RPLC generated fractionations with higher resolution and better reproducibility. Along with a unique high-pressure high-resolution separations with intelligent selection and multiplexing (PRISM) method, pictogram-level quantification of prostate-specific antigen (PSA) protein (50–100 pg/mL) was achieved in human plasma/serum (Shi et al., 2012a). Tandem application of immunoaffinity depletion and SCX fractionation has also been used and proved to boost quantitative sensitivity and precision of six low concentration spiked-in proteins (Keshishian et al., 2007). Several other chromatography techniques besides SCX and high-pH RPLC such as MCX and size exclusion chromatography (SEC) have also been employed to fulfill the needs to enrich target analytes, which are exemplified by a series of previous studies (Fortin et al., 2009; Kuhn et al., 2004).

### 6.3.3.3 Depletion of High Abundance Proteins (HAPs)

Depletion of HAPs in biomatrices is another popular experimental approach for protein enrichment. To date, this technique is mostly used in plasma/serum samples to improve the analysis of circulating markers, where the

HAPs dominate the proteome. By decreasing the protein concentration dynamic range, the target LAPs become more “visible” to MS, given that LC-MS is an abundance-dependent analytical system that usually has a 4–5 order of quantitative dynamic range. Two major approaches are employed to achieve reduced protein dynamic range: selective removal of known HAPs and nonselective reduction of protein dynamic range (Zhang et al., 2013b). Selective removal of HAPs, or immunoaffinity depletion, embraces similar principles as IC, yet antibodies targeting known HAPs (e.g., albumin, immunoglobulins) rather than the analyte proteins are used for capture. For example, in plasma, top 1 (i.e., albumin), 7, 12, or 14 HAPs can be depleted by commercially available products, leading to 90–95% of decrease in total protein masses (i.e., 10–20-fold enrichment of LAPs assuming no loss) and >10-fold lower LOD and limit of quantification (LOQ) (Liu et al., 2006; Tu et al., 2010). While recovery and reproducibility of immunoaffinity depletion could be a potential problem and has to be meticulously evaluated prior to analysis, this technique is very useful in biomarker quantification studies (Shi et al., 2012b). A tandem immunoaffinity depletion scheme combining IgY14 and SuperMix columns was reported to allow sequential depletion of approximately 60 HAPs and MAPs, resulting in approximately 100-fold LAP enrichment. The utilization of IgY14–Supermix depletion technique further boosted quantitative sensitivity (i.e., LOQ) of multiple human plasma proteins to <10 ng/mL levels (Qian et al., 2008). The IgY14–Supermix columns are currently available from Sigma-Aldrich<sup>TM</sup>. One potential problem for immunoaffinity depletion, however, is low or irreproducible protein recovery, especially when it is performed with fractionation in tandem. Nonspecific binding of target proteins and inconsistent binding to HAPs in the depletion step could both add to the variability. For example, Fortin et al. have reported that recovery of PSA protein could range from 5 to 90% when different commercial products were used for albumin depletion (Fortin et al., 2009). Hence, a thorough assessment of the depletion methods turns out to be of profound importance for the development of a reliable LC-MS workflow and shall be performed individually since the interactions among target analyte, matrix proteins, and antibodies may differ in each case.

The other HAP depletion method is global reduction of protein dynamic range via combinatorial peptide ligand library (CPLL), commercially branded as ProteoMiner<sup>TM</sup> from Bio-Rad<sup>TM</sup>. The CPLL is a repertoire of hexapeptides containing numerous protein-specific epitopes for all proteins to bind, and presumably, equimolar amounts of different species of hexapeptides are present in the library. Assuming that the binding capacity of hexapeptides against proteins is roughly the

same, similar amounts of different proteins, regardless of their abundance, will be captured by the hexapeptides and enriched. Consequently, a much larger portion of HAPs will remain unbound in the matrix and later removed compared with LAPs (Thulasiraman et al., 2005; Tu et al., 2011). This technique is employed in quite a few quantitative proteomics studies (Capriotti et al., 2012), yet applications in targeted protein quantitation are still missing.

#### **6.3.4 Methods for Protein Denaturation, Reduction, and Alkylation**

Denaturation, reduction, and alkylation serve to dissociate protein tertiary and secondary structures and transform proteins into an unfolded state. These protein pretreatment steps, if conducted properly, render cleavage sites more accessible to proteolytic enzymes, thus significantly accelerating digestion speed and improving digestion efficiency and reproducibility.

Denaturation of proteins is usually performed by addition of chaotropic agents (e.g., urea, thiourea) (Robinson and Jencks, 1965), surfactants (e.g., SDS, sodium deoxycholate (SDC), Triton X-100, NP-40) (Otzen, 2011), or both reagents, which triggers the breakdown of protein high-order structures. These denaturing reagents have to be used with particular caution; otherwise they could negatively impact the proteolytic digestion and ionization process. For example, no heating of the sample should be allowed once chaotropic agents have been added to the samples. Under high temperatures (e.g., >50°C), nonspecific carbamylation of peptide N-termini, lysine, and arginine residues can be induced (Kraus et al., 1994; Stark et al., 1960), which hampers proteolytic cleavage and peptide mass fingerprinting during SP selection. Furthermore, concentrations of chaotropic agents should be limited to a low value (e.g., <1 M) during digestion due to potential denaturing effects they could impose on proteolytic enzymes. As to surfactants, they are known to not only compromise chromatographic behavior of peptides but also cause ion suppression effects by increasing surface tensions of ESI droplets, leading to declined signal intensity (Annesley, 2003). Detergent removal is a mandatory experimental step prior to LC-MS analysis, as is included by several mainstream digestion protocols (An et al., 2015; Manza et al., 2005; Wisniewski et al., 2009). Research on more MS-friendly surfactants has been a popular research topic in the LC-MS society, and a number of surfactants with better MS compatibility have been introduced in recent years. For example, one favorable SDS substitute is SDC, an acid-labile bile salt (Coleman et al., 1979). SDC is also easy to remove by degradation via acidification, and the

degradation product produces little ion suppression effects (Masuda et al., 2008). Supplemented to the extraction/digestion buffer at a 1% w/v concentration, SDC has been found to enhance trypsin activity by up to fivefold (Masuda et al., 2008). Commercial MS-compatible surfactant products are also available on the market, such as RapiGest™ SF by Waters, ProteomeMAX™ by Promega, and PPS Silent Surfactant™ by Agilent. Most of these surfactant products are acid labile, allowing their removal by addition of acids prior to LC-MS analysis. More recently, Chang et al. have developed an acid-labile mass spectrometry-compatible degradable surfactant (MaSDeS) for tissue sample preparation. They showed that MaSDeS outperformed commercially available surfactants by overall protein solubility and the number of proteins identified in tissue proteomics, meanwhile being much easier to deplete compared with SDS (Chang et al., 2015). In addition to chaotropic agents and surfactants, 2,2,2-trifluoroethanol (TFE) is also an alternative for protein denaturation in targeted protein quantification, in which a concentration of 50% v/v TFE is used and later diluted to 5% v/v before digestion (Agger et al., 2010; Proc et al., 2010).

Protein reduction opens disulfide bridges formed between two cysteine residues to free thiols, which often helps to achieve complete unfolding of the proteins. Two categories of reagents are usually employed, including sulfur-containing substances (e.g.,  $\beta$ -mercaptoethanol, dithiothreitol (DTT)) and triple alkylated phosphines (e.g., tris(2-carboxyethyl)phosphine (TCEP)) (Dittrich et al., 2015). Several studies have reported that TCEP appears to be the better choice than  $\beta$ -mercaptoethanol or DTT because of its higher solubility and stability, as well as faster reaction rate (Getz et al., 1999; Hansen and Winther, 2009; Rebecchi et al., 2011). Subsequently, protein alkylation is performed to prevent the reformation of disulfide bridges, since thiol residues are highly reactive. Alkylation of cysteine residues is achieved by addition of alkylation reagent, and iodoacetamide (IAM) is the most commonly utilized reagent. An alternative option is *N*-ethylmaleimide (NEM), which was reported to react 20-fold faster than IAM. Alkylation needs to be carefully performed at an appropriate reagent concentration (10 mmol/L IAA as a proposed standard (Ceglarek et al., 2013)) and pH value to minimize lysine alkylation. It should also be noted that protein reduction and alkylation are to be performed in a sequential order, since DTT/TCEP could also react with IAM/NEM. A chronological overview of different combinations of denaturing, reducing, and alkylating conditions is enclosed in one review (Dittrich et al., 2015).

### 6.3.5 Methods for Proteolytic Digestion

In pursuit of efficient, reproducible, and high-throughput recovery of SPs, a number of proteolytic digestion protocols have been devised and used for LC-MS-based quantitative studies based on the considerations elaborated in previous sections. The features of several commonly practiced digestion strategies (Table 6.2) are covered in this section.

In-solution digestion is the simplest and most straightforward digestion strategy widely used for the digestion of proteins from all types of samples (De Godoy et al., 2008; Go et al., 2013; Li et al., 2004). Denaturation, reduction, alkylation, and digestion are exclusively performed in an aqueous buffer in the same tube, followed by one or multiple sample cleanup procedures (e.g., SPE) to clean up the sample and remove matrix components. In-solution digestion offers simplicity and minimal sample loss (e.g., incurred when transferring of proteins among different tubes) (Feist and Hummon, 2015). However, interferences from buffer and matrix components (e.g., chaotropic agents, surfactants, phospholipids) may be present during the digestion step, which could lead to suboptimal peptide recovery (van den Broek et al., 2013); moreover, the post-digestion cleanup step often results in peptide loss and sometimes introduces variation or errors. Some of the empirical measures to improve the performance of in-solution digestion include the use of cleavable detergents (León et al., 2013) and detergent removal by ethyl acetate-aided phase transfer (Vertommen et al., 2010). Leon et al. have performed a large-scale quantitative assessment of different combinations of conditions for in-solution digestion protocol and concluded that among all combinations tested, SDC-assisted digestion combined with phase transfer-based removal of detergents allowed the most efficient

and unbiased peptide recovery from all protein classes including membrane proteins (León et al., 2013).

In-gel digestion is another widely used digestion protocol for LC-MS-based quantification, first introduced in 1996 (Shevchenko et al., 1996). For in-gel digestion, electrophoresis-based fractionation methods are first employed to separate target proteins on a polyacrylamide gel. For targeted quantification, one-dimensional gel electrophoresis methods such as SDS-PAGE and IEF are employed in the hope of separating target proteins from most matrix interferences. In a canonical in-gel digestion protocol, gel bands containing the target proteins and their isobaric counterparts are excised from the gel, diced into small chunks (e.g., 1 mm × 1 mm), destained by addition of acetonitrile, and then undergo protein digestion (Feist and Hummon, 2015). Compared with in-solution digestion, in-gel digestion is regarded to be more robust because of its capability of significantly decreasing sample complexity and removing impurities prior to digestion (Shevchenko et al., 2007). Major drawbacks of in-gel digestion, however, are concentration-dependent and incomplete peptide recovery, cumbersome experimental procedures, and low throughput (Havlíš and Shevchenko, 2004; Speicher et al., 2000).

FASP was established by Manza et al. (2005) and later modified by Wisniewski et al. (2009). FASP is a versatile proteolytic digestion protocol that has been ubiquitously adopted for various sample types (Erde et al., 2014; Kanshin et al., 2012; Zielinska et al., 2010). Using an MW-based cutoff filter, FASP addresses the conflicts between the usage of surfactants for efficient protein extraction/denaturation and the needs to deplete surfactants from sample matrix before digestion. The filter device, described as a “proteomic reactor” (Wisniewski et al., 2009), is involved in multiple functional steps during the FASP procedures. First, the filter serves to retain protein contents and deplete salts/low MW

**Table 6.2** Prevalent proteolytic digestion methods for macromolecule biomarkers and comparison of their features.

Techniques	Protein state during digestion	Use of surfactants	Sample cleanup	Ease of operation	Length of digestion	Peptide recovery
In-solution digestion	Solubilized	Only cleavable ones	Minimal	Simple	Variable depending on the analyte	Incomplete
In-gel digestion	Fixed in gels	All types	Moderate	Laborious	Usually overnight	Incomplete
Filter-aided sample preparation (FASP)	Resuspended	All types	Good	Moderate	Usually overnight	Good yet could be irreproducible
On-pellet digestion	Precipitated/pelleted	All types	Good	Moderate	As short as 30 min	Good yet need more validation

contaminants upon centrifugation. Next, surfactant removal (e.g., SDS) can be achieved by employing a urea-containing buffer. As reported by Wisniewski et al., >99% SDS can be removed by FASP protocol, which allowed the use of an extraction buffer containing a high SDS concentration of 4% for protein extraction (Wisniewski et al., 2009). Finally, enzymes can be added directly to the filter, and peptides can be eluted in a small volume of buffer after completion of digestion, enabling concentration of the peptide solution. Moreover, high MW interfering substances can be blocked by the filter upon peptide elution. While FASP offers compelling advantages over in-solution and in-gel digestion methods, potential drawbacks such as variable peptide recoveries because of in-filter adsorption of proteins or proteolytic peptides have also been found by multiple studies (Choksaengkarn et al., 2012; Eggler et al., 2007; Liebler and Ham, 2009; Manza et al., 2005). Incomplete removal of some ionic surfactants (e.g., SDS) and interferences (e.g., phospholipids) has also been reported (Hustoft et al., 2011).

On-pellet digestion is another common proteolytic digestion method. Conventionally, on-pellet digestion combines PPT with organic solvents with pellet resolubilization and in-solution digestion, which achieves substantial predigestion sample cleanup. However, variability and sample loss can be a valid concern because of the difficulties in protein resolubilization. Vigorous vortexing and sonication, as well as meticulous evaluation of results, are necessary to ensure thorough dissociation and resolubilization of protein pellets. More recently, a novel on-pellet digestion method, coined as surfactant-aided precipitation/on-pellet digestion (SOD), has been reported (An et al., 2015; Duan et al., 2009). Typically, after spiking detergents in the sample, PPT is conducted by addition of organic solvents (e.g., acetone, chloroform/methanol, acetonitrile), and supernatant containing undesirable matrix components is discarded. The inclusion of detergents prior to precipitation greatly helps sample cleanup by improving the removal of a variety of matrix components such as phospholipids. The dual denaturing effects by surfactants and organic solvents make proteins highly accessible to proteolytic enzymes after the precipitation step, which in turn results in high-throughput and efficient digestion, for example, complete digestion of a target protein was achieved in only 30 min at 37°C, with significantly better recovery of SP than high temperature digestion; additionally, this method showed substantially higher robustness, recovery, and sensitivity than in-solution digestion, in-gel digestion, and FASP (An et al., 2015). Compared with traditional on-pellet digestion methods, SOD is performed directly on the precipitated protein pellet, and a two-step enzyme addition scheme is employed to firstly dissolve proteins back to solution and then to achieve complete protein

digestion. It was found that a protein concentration of 0.20–2.0 µg/µL was optimal for PPT (An et al., 2015). Peptide recovery after digestion significantly declined when protein concentration was <0.20 µg/µL, probably because of insufficient PPT; protein concentrations >2.0 µg/µL also resulted in low peptide recovery, and this was ascribed to the incomplete removal of surfactants and phospholipids when large chunks of precipitation were formed rapidly. Overall, on-pellet digestion methods have been demonstrated by several independent studies to bear significantly elevated peptide recoveries with high robustness compared with in-solution digestion (An et al., 2015; Ouyang et al., 2012; Yuan et al., 2012). While on-pellet digestion method appears to be an excellent alternative to offer better peptide recoveries, its applicability for both quantitative proteomics and targeted quantification studies still warrants more investigation and verification.

Besides these commonly practiced digestion protocols, alternative techniques have also been attempted to accelerate digestion rate and/or enhance digestion efficiency. Several examples of these techniques include digestion with microparticles (Guo et al., 2011) and nanoparticles (Qin et al., 2012), microwave (Segu et al., 2010; Sun et al., 2006; Ye and Li, 2012), ultrasound (López-Ferrer et al., 2005, 2008a), infrared (Wang et al., 2008a, 2008b), and high pressure (López-Ferrer et al., 2008b). These “acceleration” techniques have been thoroughly reviewed (Switzer et al., 2013), although application in large-scale biomarker analysis remains to be examined.

## 6.4 Conclusive Remarks

Efficient and reproducible sample preparation is a prerequisite for achieving accurate, precise, and robust quantification of biomarkers in biological matrices. In order to achieve this goal, the development and optimization of sample preparation procedures should be dependent on the thorough understanding of the physicochemical properties of the target biomarkers and the properties of the biological matrix. Generally speaking, methods that selectively enrich the targets while removing the matrix components to an extent sufficient for the purpose of study are preferred. For small molecule biomarkers, PPT, LLE, and SPE techniques have been regarded as effective means for the majority of biomarkers analyzed. Novel LLE- and SPE-based methods have further expanded the toolbox of bioanalysts by providing improved analyte recovery, interference removal, robustness, and affordability. For protein biomarkers, more complicated sample preparation procedures are necessary, owing to the need of protein treatment and production of SPs and the daunting challenges associated with

sensitivity, selectivity, and accuracy. In this regard, various methods and technical advancements have been demonstrated to achieve improved efficiency and reproducibility for sample cleanup, protein extraction and denaturation, and digestion. The implementation of a diversity of enrichment techniques (e.g., immunoaffinity

capture, PAGE, SCX, SISCAPA) boosts the sensitivity of protein biomarker quantification. This field is so dynamic that numerous sample preparation methods are available with new ones continuously emerging; consequently, it is essential for researchers to choose the optimal solutions in order to develop “fit-for-purpose” analytical strategy.

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## 7

## Overcome the Endogenous Levels in Biomarker Quantitation Using LC–MS

Guowen Liu

DMPK, Agios Pharmaceuticals, Cambridge, MA, USA

### 7.1 Introduction

*Biomarker* has been defined by the National Institutes of Health Biomarkers Definitions Working Group in 1998 as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group, 2001). Examples of a biomarker can be pulse, body temperature, blood pressure, or a chemical compound, and so on. The scope of this chapter is limited to a subset of biomarkers that are endogenous compounds/molecules that are generated *in vivo* and can be measured using LC–MS or LC–MS/MS. They could be either small chemical molecules or macromolecules (e.g., DNA/RNA, proteins), present at basal conditions at certain concentration levels, and may change in response to an intervention. They may be substances involved in signaling pathways or a chain of enzymatic reactions, for example, metabolic pathways. In many cases, the *in vivo* concentration of a biomarker is often an equilibrium result of its generation and disposition.

LC–MS/MS has become the method of choice for small molecule bioanalysis in the past 20 years (Xu et al., 2007) and has recently picked up its speed on protein quantitation (van den Broek et al., 2013). Although bioanalysis is a complex process, its application for drugs/drug candidates (xenobiotics) using LC–MS/MS has almost become a routine nowadays with the advancement of related technologies (easy-to-use sample preparation kits, ultrahigh pressure chromatographic technology, high-sensitivity and high-resolution mass spectrometers, etc.) and better understanding of how to apply these technologies (Xu et al., 2007). When LC–MS/MS firstly debuted on the stage of bioanalysis, there was great excitement on how powerful and simple this technology was compared with the traditional LC–UV approach. But this exuberance was quickly damped by

the discovery/observation of several factors that could severely compromise the LC–MS/MS quantitation. Matrix effect (ion suppression/enhancement by co-eluted matrix components), especially when the matrix lot-to-lot difference was present, was identified as one of the primary culprits of failing LC–MS/MS quantitation (King et al., 2000). In particular, a lot of efforts were focused on how to address the consequences resulting from matrix effect for LC–MS/MS bioanalysis (Matuszewski, 2006). For mass spectrometrists, matrix effect was originally referred to ion suppression/enhancement phenomenon from the co-eluted matrix components to the analyte of interest during the MS ionization process. In bioanalysis, matrix effect was further extended to a wide range of factors that could lead to different MS responses with the same starting concentration of analyte of interest in different lots or different types of biological matrix, such as difference in analyte stability, protein binding, analyte extraction recovery, and MS ionization. All these factors could lead to assay variation/inaccuracy if not corrected. Therefore, when scientists try to develop a quantitative bioanalytical method, substantial efforts have been put on addressing matrix effect. Not surprisingly, the default strategy is to match the blank matrix used for calibration standards with the unknown samples. This strategy is designed to minimize the difference between unknown samples and the calibration standards on matrix background. This can be easily achieved for exogenous compounds through using the same biological matrix, for example, using blank human plasma to prepare calibration standards to quantify unknown human plasma samples. However, in reality, biological matrix is highly complicated and we seldom obtain two identical lots of matrix. Therefore, matching blank matrix alone still does not always (almost never) address the matrix effect. For this reason, a second strategy using an internal standard (IS) (Liu et al., 2013) is routinely used, which can track the analyte of

interest from sample preparation to LC-MS detection with regard to variations on analyte stability/solubility, protein binding, extraction recovery, chromatographic separation, and MS ionization. In recent years, stable isotope-labeled (SIL) version of the analyte has been commonly used as an ideal IS to track all these steps, which has been demonstrated to be an effective strategy for compensating matrix effect in a broad sense (Liu et al., 2010). For pharmacokinetic (PK) assays, since it is fairly easy to combine both strategies, an approach of matching blank matrix with an SIL-IS is widely used and has become the default option. However, when it comes to biomarkers, it is a different story. This is mainly because biomarkers are endogenous compounds and they are presenting at certain concentration levels at basal conditions. This makes a true blank matrix (the same matrix without the analyte of interest) difficult to obtain. Therefore, how to deal with endogenous levels has become a hot topic for biomarker bioanalysis. In this chapter, we will discuss in detail the challenges related to endogenous levels of biomarkers for LC-MS/MS bioanalysis and review strategies used by scientists to address these challenges. To contrast small molecule PK assays, we will focus on small molecule biomarkers using LC-MS/MS. Most importantly, we will analyze the fundamentals of this issue of endogenous levels to understand why we need or why we may not need to have authentic blank matrix and provide general examples of how to mitigate this problem.

## 7.2 How Does Matrix Effect Affect Quantitation?

To have a better understanding of this problem, it is always good to look at the basics on quantitative analysis using LC-MS/MS with an IS. For LC-MS/MS assays, the calibration curve can often be expressed by the following simple equation:

$$Y = \alpha X + \beta \quad (7.1)$$

*Y*: Measured instrument response ratio of analyte of interest to IS

*X*: Analyte concentration

$\beta$ : Intercept (instrument response ratio in blank matrix sample)

$\alpha$ : Calibration curve slope

The deciding factor for accurately predicting concentration of unknown samples is the measured *Y*.

$$Y = \frac{\text{Resp}(A)}{\text{Resp}(IS)}; A, \text{analyte}; IS, \text{internal standard} \quad (7.2)$$

$$\text{Resp}(A) \propto V_A \times C_A \times \text{Rec\%} \times V_i \times I_A \times D_A \quad (7.3)$$

$$\text{Resp}(IS) \propto V_{IS} \times C_{IS} \times \text{Rec\%} \times V_i \times I_{IS} \times D_{IS} \quad (7.4)$$

$V_A$ : Aliquot volume of analyte solution/sample

$V_{IS}$ : Aliquot volume of IS solution

$C_A$ : Concentration of analyte

$C_{IS}$ : Concentration of IS

$\text{Rec\%}$ : Overall recovery during sample preparation

$V_i$ : Injection volume (the same for analyte and IS)

$I_A$ : Instrument-dependent ionization factor of analyte

$I_{IS}$ : Instrument-dependent ionization factor of IS

$D_A$ : MS detection factor of analyte

$D_{IS}$ : MS detection factor of IS

Analyte or IS will be separated from each other in mass spectrometer. This MS detection factor covers every step in the mass spectrometer after ionization. Since most MS detectors have a linear response range,  $D_A$  and  $D_{IS}$  could be different if one of them is outside the linear response range.  $D_A$  and  $D_{IS}$  could also be different if the fragmentation pattern is different due to isotopic effect. Therefore, Equation 7.2 can be written as:

$$Y = \frac{V_A \times C_A \times \text{Rec\%} \times V_i \times I_A \times D_A}{V_{IS} \times C_{IS} \times \text{Rec\%} \times V_i \times I_{IS} \times D_{IS}} \quad (7.5)$$

When a perfect IS is used, it can track the process of recovery, injection, and ionization; then Equation (7.5) can be simplified to the following:

$$Y = \frac{V_A \times C_A \times D_A}{V_{IS} \times C_{IS} \times D_{IS}} \quad (7.6)$$

As we can tell from Equation (7.6), the components solely related to matrix become irrelevant when a perfect IS is used. A SIL version of the analyte of interest can often serve for such purpose with rare exceptions (Jemal et al., 2003; Wang et al., 2007; Zhang and Wujcik, 2009). In cases when an SIL-IS could not serve as a perfect IS, an extensively labeled deuterated IS was usually involved. It was not because that the SIL-IS could not track the analyte during the sample preparation step, but due to a slight separation between the deuterated IS and the authentic analyte caused by isotopic effect leading to differential ion suppression/enhancement (Jemal et al., 2003; Wang et al., 2007; Zhang and Wujcik, 2009). This could be resolved by modifying HPLC conditions to make sure two compounds were eluted out close enough (Zhang and Wujcik, 2009) or by minimizing the amount of matrix injected to LC-MS/MS if sensitivity allows (Wang et al., 2007). Other non-HPLC-related isotopic effects (e.g., fragmentation difference, detection saturation on one but not on the other (Liu et al., 2010)) could also be addressed by designing the experiment carefully. Therefore, in theory, most variations/differences during

sample preparation and LC-MS analysis caused by matrix effects, such as compound stability, solubility, extraction recovery, and ionization enhancement or suppression, can be addressed by using an SIL-IS with good experimental design, which makes matching the blank matrix no longer critical. Of course, there will always be some exceptions. We will discuss how to identify and address the exceptions when they do happen later on. It is also worth to mention that some basic parameters should always be evaluated for any bioanalytical method, such as compound stability/solubility during sample collection, shipment and storage, and protein binding difference across different samples, which may not be effectively addressed by using SIL-IS.

### 7.3 Commonly Used Strategies

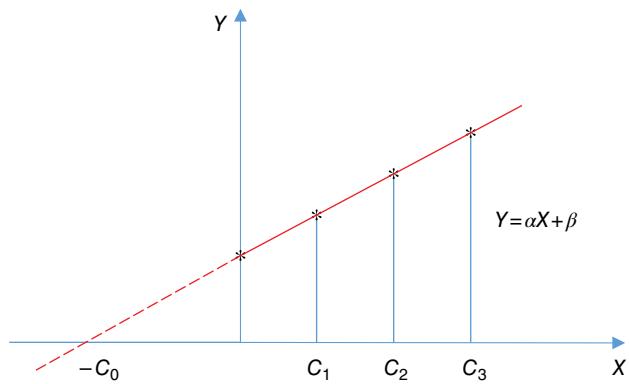
To address the endogenous level challenge, scientists have come up with different strategies. Specific to LC-MS/MS assays, this has been reviewed comprehensively by van de Merbel (2008) and Jian et al. (2013a, 2013b). Depending on how the calibration standards are prepared, three different approaches have commonly been used:

- 1) Authentic analyte in authentic matrix: Spiking authentic analyte to authentic matrix to prepare calibration standards
- 2) Surrogate analyte in authentic matrix: Spiking surrogate analyte to authentic matrix to prepare calibration standards (surrogate analyte approach)
- 3) Authentic analyte in surrogate matrix: Spiking authentic analyte to a surrogate matrix to prepare calibration standards (surrogate matrix approach)

In the following paragraphs, we will briefly describe each approach and propose a detailed protocol for each.

#### 7.3.1 Authentic Analyte in Authentic Matrix (Standard Addition)

The “authentic analyte in authentic matrix” approach can be represented by the “standard addition” approach. In this chapter, we will exclude those cases in which a true blank authentic matrix is possible by either screening for different lots of matrix or by manipulation (e.g., charcoal stripped) of authentic matrix to make sure that the endogenous level can be controlled at below 20% of the desired LLOQ. In these cases, since endogenous level is no longer a challenge, the strategies/practices for regular PK assays can be followed and they will not be discussed here. For the standard addition approach, as shown in Figure 7.1, different amounts of analyte of interest are spiked into the unknown sample to achieve



**Figure 7.1** Scheme of standard addition.

different final spiked concentrations (e.g.,  $C_1$ ,  $C_2$ ,  $C_3$ ). Preferably at least three different concentrations are spiked and all four samples are then analyzed. A calibration curve is constructed based on the four samples and the intercept on the  $X$ -axis (absolute value) is measured as the endogenous level ( $C_0$ ). A standard protocol is suggested as below (protocol A). This approach can be used in most situations and has been reported as the ultimate approach to verify if other approaches are valid (Jones et al., 2012; Ghassabian et al., 2015). The major challenges related to this approach are its throughput, workload, and the requirement of large sample volume.

Protocol A (example):

- 1) Split unknown samples into four different aliquots.
- 2) Spike 0, low ( $C_1$ ), middle ( $C_2$ ), and high ( $C_3$ ) levels of authentic analyte.
- 3) Add SIL-IS working solution to each sample and analyze all samples together.
- 4) Generate a calibration curve using all four samples and obtain an equation; assume the equation is  $Y = \alpha X + \beta$ .
- 5) The endogenous level will be  $C_0 = \beta/\alpha$ .

#### 7.3.2 Surrogate Analyte in Authentic Matrix

When the standard addition is not practical to address the endogenous level problem due to its limitations, there are other approaches to deal with the problem of incompatible combination of “authentic biological matrix + endogenous analyte (authentic analyte).” Changing one of the two components naturally comes into play. SIL compounds, such as  $^2\text{H}$ -,  $^{13}\text{C}$ -,  $^{15}\text{N}$ -, and  $^{18}\text{O}$ -labeled version of the authentic analyte, normally bear almost identical chemical and physical properties as their naturally occurring compounds, only differing by molecular weight (mass). Fortunately, mass spectrometer can easily differentiate molecules by their masses. Therefore, using a SIL version of the analyte of interest (which does not exist in the authentic biological matrix) to serve as a surrogate for the authentic analyte to prepare calibration standards for biomarker analysis

using LC-MS/MS becomes a logical and popular idea. The expectation is that the extraction recovery, chromatographic retention, and detection properties of the surrogate and authentic analytes are identical or at least that possible differences are small and constant. Specifically, a SIL surrogate analyte is used instead of the authentic analyte in the calibration standards. QCs can be prepared using surrogate analyte too, but at least one level of QC should be prepared using the authentic analyte. A third compound is added to both calibration and study samples to serve as IS. The IS has to differ from the surrogate analyte by mass (or at least a distinct MRM) and can be either a non-endogenous structural analog or another SIL form of the analyte. This approach was early tried out by Li and Jemal in 2003 (Jemal et al., 2003; Li and Cohen, 2003). It has been since widely adopted and practiced in many case studies (see a summary in Table 7.1). In practice, people did not automatically assume the equivalence between the surrogate analyte and the authentic analyte. A response factor (RF) was often recommended to be established to bridge the surrogate analyte and the authentic analyte (Li and Cohen, 2003; Jian et al., 2010; Leonard et al., 2014; Ongay et al., 2014; Liu et al., 2016). A typical protocol for this approach based on published literature (see Table 7.1) is as follows, assuming the analyte of interest is compound XYZ:

Protocol B (example):

- 1) Obtain a SIL ( $^2\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{18}\text{O}$ , etc., -XYZ.) version of the analyte of interest SIL-XYZ-1.
- 2) Obtain another SIL version of the analyte of interest SIL-XYZ-2 as IS (if possible).
- 3) Optimize MS conditions for XYZ, SIL-XYZ-1, and SIL-XYZ-2 and develop an LC-MS/MS method for all three compounds. One important challenge always needed to be taken care of is the isotopic cross-talking when different versions of SIL compound are used in one assay. One has to make sure that there is either no isotopic cross-talking in MRM or at least the interferences are insignificant and will not impact the final quantitation.
- 4) Spike SIL-XYZ-1 to the authentic biological matrix to prepare calibration standards and analytical QCs (low, middle, high).
- 5) Spike SIL-XYZ-1 and XYZ in a neat solution to prepare samples at different concentration levels (equimolar of SIL-XYZ-1 and XYZ in each level) across the standard curve range (can be at exact concentration levels as the calibration standards).
- 6) Obtain a big pool of authentic matrix and measure the endogenous level ( $\alpha$  ng/mL) using standard addition approach (see Section 7.3.1).

- 7) Prepare authentic QCs by spiking different amount of XYZ to the above authentic matrix to prepare LQC (endogenous level:  $\alpha$  ng/mL), MQC ( $\alpha + b$  ng/mL), and HQC ( $\alpha + c$  ng/mL).
- 8) Calculate an MS response factor (RF) between XYZ and SIL-XYZ-1 by averaging the ratios of MS responses between XYZ and SIL-XYZ-1 in all levels of neat solution samples containing both compounds:

$$RF = \text{Avg} \left( \frac{MS_{(\text{SIL-XYZ-1})}}{MS_{(\text{XYZ})}} \right) \quad (7.7)$$

Predefined acceptance criteria will be used to accept or reject the RF at a certain level as well as accept or reject a certain run. For example, when the RF from a certain neat solution sample deviates more than 15% (20% at LLOQ) of the mean, that RF is excluded from the final calculation; when more than 25% of the total testing neat samples are excluded, the run is rejected.

- 9) Generate a calibration curve based on the surrogate analyte:

$$Y = \alpha X + \beta \quad (\text{where } Y = MS_{(\text{SIL-XYZ-1})} / MS_{\text{IS}})$$

$$X = \left( MS_{(\text{SIL-XYZ-1})} / MS_{\text{IS}} - \beta \right) / \alpha \quad (7.8)$$

- 10) Convert the MS response of XYZ in unknown sample ( $MS_{\text{XYZ}}$ ) to MS response of SIL-XYZ-1 ( $MS_{(\text{SIL-XYZ-1})}$ ) by applying the response factor  $RF$ .  $MS_{(\text{SIL-XYZ-1})} = RF \times MS_{\text{XYZ}}$  and plug in the converted signal to Equation (7.8) to calculate the authentic analyte concentration.
- 11) Use analytical QCs (surrogate analyte) to accept or reject a run.
- 12) Use authentic QCs (spiked authentic analyte to authentic matrix) to evaluate analyte stability and parallelism between surrogate analyte and authentic analyte.

Table 7.1 summarizes published case studies up to date on using surrogate analyte approach by LC-MS/MS for biomarker quantitation. By reviewing the reported case studies, surrogate analyte approach has been demonstrated to be an effective solution to address the endogenous level issue related to biomarker quantitation in almost every common biological matrix, such as plasma, urine, blood, CSF, and tissue homogenate. Most methods can be validated by following regulatory guidance (US FDA, 2001; EMA, 2011) and industrial best practices (Viswanathan et al., 2007) used for PK assays. Either an

**Table 7.1** Examples of reported biomarker assays using surrogate analyte approach.

Analyte	Surrogate analyte	Internal standard	Matrix	LLOQ	Val./Qual*	RF**	Reference	Note
KIC <sup>a</sup>	D <sub>3</sub> -KIC	Ketoisocaproic acid (analog)	Rat plasma Brain homogenate	10 ng/mL	Val.	0.95–1.06	Li and Cohen (2003)	No QC prepared with authentic analyte in authentic matrix was tested
MVA <sup>b</sup>	D <sub>3</sub> /D <sub>4</sub> -MVA	D <sub>7</sub> -MVA	Human plasma Human urine	0.5 ng/mL 25 ng/mL	Val.	N/A	Jemal et al. (2003)	
Sorbitol	<sup>13</sup> C <sub>6</sub> -sorbitol	D <sub>2</sub> -sorbitol	Human erythrocyte	50 (sorb.)	Val.	N/A	Liang et al. (2005)	
Fructose	<sup>13</sup> C <sub>6</sub> -fructose	D <sub>3</sub> -fructose	Nerve tissue	250 (fruc.)				
HA	<sup>13</sup> C <sub>6</sub> -HA	L-phenylalanine, ring-D <sub>5</sub>	Monkey urine	0.25 µg/mL 0.1 µg/mL	Qual.	N/A	Penner et al. (2010)	No bridging between surrogate and authentic analyte
BA <sup>c</sup>	<sup>13</sup> C <sub>6</sub> -BA							
AEA	D <sub>4</sub> -AEA	D <sub>8</sub> -AEA	Human plasma	0.1/0.5/0.5 ng/mL	Val.	AEA:1.08	Jian et al. (2010)	No RF applied because RF were determined to be 1.08, 1.09, and 0.91 for AEA, OEA, and PEA, respectively, very close to the theoretical value of 1.00
OEA	D <sub>4</sub> -OEA	D <sub>2</sub> -OEA				OEA:1.09		
PEA <sup>d</sup>	<sup>13</sup> C <sub>2</sub> -PEA	D <sub>4</sub> -PEA				PEA:0.91		
DES	<sup>18</sup> O <sub>8</sub> -DES	D <sub>4</sub> -DES	Human urine	1 nM/1 nM	Val.	IDS ~1 DES: (0.5–1.25)	Ongay et al. (2014)	SA and SM are comparable
IDS <sup>e</sup>	<sup>18</sup> O <sub>8</sub> -IDS	D <sub>4</sub> -IDS						
Creatinine	D <sub>3</sub> -creatinine	<sup>13</sup> C <sub>3</sub> D <sub>4</sub> <sup>15</sup> N-creatinine	Human urine	10 ng/mL	Val.	Diff% < 0.7%	Leonard et al. (2014)	
NAAG <sup>f</sup>	D <sub>5</sub> -NAAG	D <sub>8</sub> -NAAG	Rat brain, plasma and CSF	Rat brain: 1.0 nmol/g; rat plasma/CSF: 0.01 nmol/mL	Val.	N/A	Kinoshita et al. (2015)	
CA	D <sub>4</sub> -CA	Dehydrocholic acid	Serum and plasma	4.9 nM	Qual.	N/A	Zheng et al. (2016)	Discovery level method
DCA	D <sub>4</sub> -DCA							
LCA	D <sub>4</sub> -LCA							
GUDCA	D <sub>4</sub> -GUDCA							
GCA	D <sub>4</sub> -GCA							
TCDCA	D <sub>5</sub> -TCDCA							
TCA <sup>g</sup>	D <sub>5</sub> -TCA							
NAD+	<sup>13</sup> C <sub>5</sub> -NAD+	<sup>13</sup> C <sub>5</sub> D <sub>3</sub> -NAD+	Human blood	0.25 µg/mL	Val.	1.01	Liu et al. (2016)	No RF applied

Notes:

\* Qual, method was qualified following fit-for-purpose predefined criteria; Val., method was validated according to regulatory guidance.

\*\* RF, response factor: MS response of surrogate analyte to MS response of authentic analyte.

<sup>a</sup> KIC, a-ketoisocaproic acid.

<sup>b</sup> MVA, mevalonic acid.

<sup>c</sup> BA, benzoic acid; HA, hippuric acid.

<sup>d</sup> AEA, anandamide (AEA); OEA, oleoylethanolamide; PEA, palmitoylethanolamide.

<sup>e</sup> DES, desmosine; IDS, isodesmosine.

<sup>f</sup> NAAG, N-acetyl-L-aspartyl-L-glutamic acid.

<sup>g</sup> CA, cholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GUDCA, glucoursoodeoxylcholic acid; LCA, lithocholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid.

MS RF established between the surrogate analyte and the authentic analyte or QCs prepared with authentic analyte in authentic matrix with endogenous levels corrected or both approaches were used to bridge the surrogate analyte and authentic analyte. In cases when an RF was established, the value close to 1 was observed in most cases with one exception (Ongay et al., 2014). In this specific case (Ongay et al., 2014), the MS RF of  $^{18}\text{O}_8\text{-DES/DES}$  (please refer to each table for all abbreviations used across the main text) was found to be variable from time to time. Although it was constant at all concentration levels on a certain day, it varied after instrument shutdown or long inactivity periods. At the same time, the RF of another pair of compounds  $^{18}\text{O}_8\text{-IDS/IDS}$  was constant and close to 1 at all times. The exact cause of variable RF was not identified. However, the accuracy of authentic analyte QCs was found to be excellent after applying the RF, which demonstrated that the surrogate analyte approach could still work well even the RF was variable and not close to 1. On the other hand, if an RF was not established and the signal between the surrogate analyte and the authentic analyte was not corrected by applying an RF, the measured concentration of the authentic analyte against a calibration curve based on the surrogate analyte could be inaccurate, as demonstrated in one example (Kindt et al., 2004). In that report, it was mentioned that a surrogate analyte approach was tried without success due to unacceptable accuracy for matrix-based QC samples. It was suggested by the authors that a difference in the signal-to-noise ratios between the SRM channels of the analyte and the SIL-IS was the possible cause. However, it was not clear why a signal-to-noise ratios difference between the analyte and its SIL-IS would cause such a failure. There were not enough details to draw a conclusion about what was the exact reason for the failure of the surrogate analyte strategy. On the other hand, there was no concept of RF mentioned in the publication (Kindt et al., 2004), and therefore the MS response difference between the surrogate analyte and the authentic analyte might not be corrected, which could be the real reason behind the failure of the surrogate analyte approach. Nonetheless, when a surrogate analyte approach is adopted, it is strongly recommended to establish an RF in each run and correct the MS response of the authentic analyte as necessary to bridge it with that of the surrogate analyte. It is also recommended to test QCs prepared by spiking authentic analyte in authentic matrix. Such QCs will not only help confirm the validity of the surrogate analyte approach but will also facilitate to detect any stability issue (Jian et al., 2010). Since authentic analyte (biomarker) is an endogenous compound, its stability could be impacted by two factors: generation and degradation. As discussed earlier, biomarker *in vivo* concentration is

usually an equilibrium result of its generation and degradation. Both processes could still happen *ex vivo*. Therefore, it is always necessary to test QCs with authentic analyte in authentic matrix for stability evaluation. It is also worth to mention that the stability of the authentic analyte could be significantly different in fresh matrix than that in aged matrix (Jian et al., 2010; Onorato et al., 2014) due to difference in some enzyme activities. For all these reasons, stability evaluation only using surrogate analyte in authentic matrix is discouraged because it will not cover the *ex vivo* generation of the authentic endogenous analyte from its precursor(s). To sum up, the surrogate analyte approach should always work with proper experiment designs. However, it should be recognized that there are indeed some operational challenges with this approach. For example, several post-acquisition data (MS responses) processing steps, such as establishing the RF, converting MS response of the authentic analyte to the response of the surrogate analyte, and plugging in the converted MS response back to the system, will usually have to be handled outside the validated bioanalytical dataflow. This imposes some challenges for case studies requiring fully validated assays (Booth, 2011).

### 7.3.3 Authentic Analyte in Surrogate Matrix

Another approach to address the incompatibility of “authentic matrix + authentic endogenous analyte” is to use a surrogate matrix, a matrix/solution that is different from the authentic matrix and contains no analyte of interest. Authentic analyte is spiked in a surrogate matrix to prepare calibration standards and analytical QCs. As we have discussed in Section 7.1, an SIL-IS becomes readily available and has been widely used in many LC-MS/MS assays for absolute quantitation. Also as having been extensively discussed in Section 7.2, an SIL-IS can serve as a perfect IS with some rare exceptions (Jemal et al., 2003; Wang et al., 2007; Liu et al., 2010) to track the analyte from sample preparation to MS ionization, which makes matrix-related factors not critical anymore with regard to assay accuracy and precision. These exceptions have been discussed in Section 7.2 and can be easily identified and mitigated with proper experiment design. In other words, the impact on quantitation results due to different lots or different types of matrix becomes very small and negligible in most cases once an SIL-IS is used. Therefore, when an SIL-IS is available, using surrogate matrix to deal with the endogenous level issue for biomarker quantitation becomes a logical and viable approach. As shown in Table 7.2, surrogate matrix approach has been used successfully in common biological matrix such as plasma, serum, blood, cerebrospinal fluid (CSF), and tissue homogenate for many types of analytes. A typical protocol for the “authentic analyte in

**Table 7.2** Examples of reported biomarker assays using surrogate matrix approach.

Analyte	Surrogate matrix	Internal standard	Authentic matrix	LLOQ	Val. /Qual*	Authentic QC tested	Reference	Note
Myo-inositol	Water	D <sub>6</sub> -myo-inositol	Rat brain tissue homogenate	0.1 µg/mL	Val.	Yes	Kindt et al. (2004)	Surrogate analyte approach was also tried but failed
Mia-114	Charcoal-stripped rabbit plasma	D <sub>4</sub> -Mia-114	Human plasma	2 ng/mL	Val.	Yes	Catz et al. (2005)	A method validated in rabbit plasma was used to support human plasma
NIC		D <sub>4</sub> -NIC		8 ng/mL				
NAM <sup>a</sup>		D <sub>4</sub> -NAM		75 ng/mL				
Uric acid, xanthine, hypoxanthine	Phosphate solution	Analog IS	Human serum	10 µM 0.2 µM 0.2 µM	Val.	Yes	Cooper et al. (2006)	LC-UV method
Hepcidin	Rabbit serum	Analog IS (CGRP)	Human serum Mouse serum	0.25 ng/mL 1.0 ng/mL	Qual.	Yes	Murphy et al. (2007)	Human hepcidin is different from rabbit hepcidin
Hepcidin	Rabbit serum	<sup>13</sup> C <sub>9</sub> <sup>15</sup> N-hepcidin	Human serum	2.5 ng/mL	Val.	Yes	Li et al. (2009)	Rabbit serum contains no human hepcidin
Mevalonic acid	Water	SIL-IS (deuterated)	Human serum	0.5 ng/mL	Val.	Yes	Waldron and Webster (2011)	N/A
Oxytocin	Human plasma diluted with water (1:6, v/v)	<sup>13</sup> C <sub>6</sub> <sup>15</sup> N-oxytocin	Rat plasma Human plasma	50.0 pg/mL 1.0 pg/mL	Val.	Yes	Zhang et al. (2011)	Human endogenous level is very low. Nonspecific binding is an issue in pure water
Glycine	Artificial CSF	SIL-IS ( <sup>13</sup> C <sub>2</sub> , <sup>15</sup> N-glycine)	Human CSF	50 ng/mL	Val.	No**	Wilson et al. (2011)	Surrogate matrix and surrogate analyte approaches are equivalent
LTB4 <sup>b</sup>	Protein buffer (10 mg/mL HSA in PBS)	<sup>13</sup> C <sub>2</sub> -LTB4	Human sputum	0.2 ng/mL	Qual.	Yes	Jian et al. (2013a, 2013b)	Limited authentic blank matrix supply
PLP <sup>c</sup>	2% BSA in PBS	D <sub>3</sub> -PLP	Human blood	5 ng/mL	Val.	Yes	Ghassabian et al. (2015)	Standard addition to obtain endogenous level in blank authentic matrix
Neu5Ac	5% BSA	D <sub>3</sub> -Neu5Ac	Human plasma	10/25 ng/mL	Qual.	Yes	Shi et al. (2015)	Although recovery was significantly different between surrogate matrix and authentic matrix, the quantitation results were not affected because an SIL-IS was used
ManNAc <sup>d</sup>		<sup>13</sup> C-D <sub>3</sub> -ManNAc						
24(S)-HC <sup>e</sup>	5% BSA (plasma) 2.5% HP-β-CD <sup>f</sup> (CSF)	D <sub>7</sub> -24(S)-HC	Human plasma Human CSF	1.0 ng/mL 0.025 ng/mL	Val.	Yes	Sidhu et al. (2015)	Nonspecific absorption issue in CSF was addressed by adding 2.5% HP-β-CD into CSF samples

Notes:

\* Qual. method was qualified following fit-for-purpose pre-defined criteria; Val. method was validated according to regulatory guidance.

\*\* QC in surrogate matrix only, but standard addition approach was used to confirm the accuracy.

<sup>a</sup> Mia-114, myristyl nicotinate; NAM, nicotinamide; NIC, nicotinic acid.

<sup>b</sup> LTB4, leukotriene B4.

<sup>c</sup> PLP, pyridoxal-5'-phosphate.

<sup>d</sup> ManNAc, *N*-acetylmannosamine; Neu5Ac, *N*-acetylneurameric acid.

<sup>e</sup> 24(S)-HC, 24(S)-hydroxycholesterol.

<sup>f</sup> HP-β-CD, 2-hydroxypropyl-β-cyclodextrin.

surrogate matrix” is proposed as follows (assuming the compound of interest is compound XYZ):

Protocol C (example):

- 1) Obtain a suitable surrogate matrix to ensure suitable solubility and similar nonspecific binding (synthetic matrix is commercially available for common matrix, such as SeraSub® for serum and UriSub® for urine from CST Technologies, Inc.).
- 2) Obtain an SIL version of analyte of interest SIL-XYZ (avoid an extensively deuterated version, if possible, to minimize isotopic effect on chromatography).
- 3) Prepare standard curve and analytical QC s in surrogate matrix by spiking authentic analyte.
- 4) Obtain a big pool of authentic matrix and measure the endogenous level using standard addition approach (assume the endogenous level is  $a$  ng/mL).
- 5) Prepare authentic matrix QC s by spiking authentic analyte to obtain three levels of QC s: LQC ( $a$  ng/mL), MQC ( $a + b$  ng/mL), and HQC ( $a + c$  ng/mL).
- 6) Use analytical QC s to accept the run and authentic QC s to establish parallelism and stability.

As shown in Table 7.2, most methods could be validated following criteria defined in regulatory guidance (US FDA, 2001; EMA, 2011) as that for PK assays. In most cases, an SIL-IS was used, and authentic QC s by spiking authentic analyte in authentic matrix were evaluated during method validation or qualification. As for the case study of glycine analysis in human CSF (Wilson et al., 2011), both surrogate matrix and surrogate analyte approaches were tested and equivalent results were obtained. As for the case study of quantitation of Neu5Ac and ManNAc in human plasma (Shi et al., 2015), 5% BSA aqueous solution was used as the surrogate matrix. Although the recovery of Neu5Ac was only about 70% in human plasma, but was more than 113% in 5% BSA, the accuracy of the assay in authentic QC s in human plasma was within 8.3% from their nominal values with inter-assay precision less than 4.9%. This suggested that the SIL-IS perfectly eliminated the impact from the significant difference in recovery between the surrogate matrix and the authentic matrix. It is worth emphasizing that authentic QC s (authentic analyte in authentic matrix) are essential to ensure the surrogate matrix approach is working. They are not only used to establish parallelism. “Parallelism” in ligand binding assays usually refers to that dilution of a sample does not result in biased measurements of the analyte concentration. Here it refers to the equivalence of using “authentic matrix” or “surrogate matrix” in preparing calibration curves. For example, if we constructed two calibration curves by “spiking authentic analyte in authentic matrix” and “spiking authentic analyte in surrogate matrix,” their slopes should be the same not only to be deemed parallel between the

surrogate matrix and the authentic matrix but also to gauge the stability concerns that are specific to authentic matrix as discussed in Section 7.3.2. Surrogate matrix approach bears many advantages. Compared with the surrogate analyte approach, it only requires one SIL-IS and can follow existing workflow for PK assays for data processing. However, it does have some concerns on different analyte solubility and nonspecific adsorption between the surrogate matrix and authentic matrix. These factors need to be taken care of during surrogate matrix selection and method development. It is also worth to mention that when surrogate matrix approach is used, the absolute sensitivity (absolute MS response) of the analyte and IS could be significantly different in surrogate versus authentic matrix, although the response ratio may remain the same. Very often, absolute sensitivity of both analyte and IS is better in surrogate matrix when a PBS buffer or a simple organic solution is chosen as the surrogate matrix. This does have an impact on deciding the LLOQ of the method.

## 7.4 Discussions and Future Perspectives

In this chapter, we have discussed the fundamental aspects and historical reasons of requiring matching biological matrix for calibration standards with unknown samples in LC-MS/MS bioanalysis. We have also discussed why this is a challenge for biomarker quantitation because of their endogenous nature. It all comes down to the reality of lacking truly blank authentic matrix (biological matrix matching to the unknown samples) to prepare calibration standards for biomarker quantitation following traditional thinking. However, as discussed in this chapter, the absolute need to match the biological matrix used for calibration standards with the unknown samples becomes debatable when a good SIL-IS is available. The historical justification of requiring matched blank matrix is to minimize the differences between calibration standards and unknown samples. By doing this, one is trying to reduce the observed variations on analyte extraction recovery and MS ionization across different lots or different types of biological matrix. There is no question that these variations could be reduced to a certain level by matching biological matrix. However, these benefits can be easily achieved and outpaced by using an SIL-IS. In reality, ever since the common use of an SIL-IS in PK assays, there was less and less report about bioanalytical assay failures that are due to inconsistent analyte recovery during sample preparation or matrix ion suppression/enhancement during the ionization step. This was because an SIL-IS can easily track these variations

and cancel out any difference across samples. Not surprisingly, as reviewed in Section 7.3.3, when an SIL-IS was available, the endogenous level challenge could be easily addressed by using a surrogate matrix. The author strongly recommends use of this approach whenever possible because of its simplicity and scientific justifications discussed in this chapter. On the other hand, as reviewed in Section 7.3.2, the “surrogate analyte in authentic matrix” approach is also a viable choice, especially when two SIL versions of the analyte of interest are available. Challenges related to this approach are the availability of SIL versions of analyte and post-acquisition data processing. In summary, when at least one SIL

version of the analyte of interest is available, the challenge of endogenous levels can be addressed by either “authentic analyte in surrogate matrix” or “surrogate analyte in authentic matrix.” However, it should be emphasized here that it is very critical to test authentic QCs to demonstrate sample stability and equivalence between surrogate analyte and authentic analyte or equivalence between surrogate matrix and authentic matrix, no matter which approach is adopted. In case when no SIL analog is possible, the endogenous level challenge becomes more complicated. A standard addition approach may be required for absolute quantitation, if notable lot-to-lot matrix effect difference is observed.

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## **Part II**

### **Challenges and Approaches**

**8****Sample Collection for Targeted Biomarker Quantitation by LC–MS**

*Yuzhong Deng and Xiaorong Liang*

*Drug Metabolism and Pharmacokinetics, Genentech, South San Francisco, CA, USA*

**8.1 Introduction**

There is growing evidence that endogenous molecules, such as bioactive small molecules, anabolic products, microRNAs (miRNA), peptides, and proteins, can be utilized as biomarkers to facilitate drug discovery and development process (Atkinson et al., 2001; de Carvalho et al., 2016; Lee et al., 2006; Surinova et al., 2011; Wagner et al., 2007). Appropriate application of biomarker data in preclinical and clinical studies can enable more predictive and reliable decision making. Accurately measuring these biomarkers have great impact on these studies. One of the major components of total laboratory quality is the preanalytical phase. This is the phase that occurs before the sample is analyzed using a specific biomarker testing method. It comprises biologic, procedure-related, and *in vitro* variables that can alter the measured level of the analyte in the specimen. Failure to control the preanalytical phase introduces a potential source of error and variation in the data and can lead to misinterpretation of the biomarker response. Studies show that preanalytical procedures, including patient preparation, specimen acquisition, handling, and storage, can account for up to 93% of laboratory errors within the entire diagnostic process (Lippi et al., 2006).

Among the preanalytical variables, sample collection protocols and storage conditions for the biological samples are arguably the most important. In addition, biomarker samples usually are collected along with the pharmacokinetic (PK) samples during the trial. It is very important to understand the differences between the PK assays and the biomarker assays and the conditions required for sample collection of these two types of samples. Practically, it is always easier if the sample collected for the PK analysis can also be used for biomarker analysis. But sample collection conditions need to be evaluated to ensure that stability for all analytes of interest in the single sample can be used for both PK and biomarker

analyses. Alternatively, separate samples can be collected for PK and biomarker analyses. Most of the clinical protocols prefer to use separate samples for PK and biomarker analyses since the requirement of the assays is different. PK assays are usually validated following the good laboratory practice (GLP) guidance, while many of the biomarker assays are only relative quantitative and for exploratory purpose. Using separate samples for PK and biomarker analyses can maintain a cleaner record between the GLP and non-GLP environments.

Many types of biomarkers are designed by nature to have only fleeting stability. Therefore, focusing on maintaining analyte integrity from sample collection to storage and further on to analysis is of utmost importance for successful bioanalytical support. In this chapter, we discuss the variables that should be experimentally evaluated to optimize sample collection for biomarkers that include the following: timing of sample collection, matrix type, collection method and preservation, and sample storage stability.

**8.2 Timing of Biomarker Sample Collection**

Biomarker levels can fluctuate annually, seasonally, monthly, daily, or even hourly. For example, there is a difference in hormone and various metabolite levels detected in the first morning urine in comparison with subsequent collections (Hunter, 1997; Landi and Caporaso, 1997; Saracci, 1997). Multiple sample collection time points are often necessary in order to obtain the true time course for the relationship of the exposure to development of the outcome and to establish causal associations (Hunter, 1997). For estrogen and progesterone, their levels vary widely across the menstrual cycle in premenopausal women. Depending on the study population and resources, premenopausal women may be asked

to collect their samples at specific times in the menstrual cycle, when sex hormones are relatively stable from day to day, and return a postcard with the date of their next menstrual cycle so the investigator can track when the sample is collected during their menstrual cycle (Tworoger and Hankinson, 2006). Some steroids, like dehydroepiandrosterone (DHEA), have strong circadian variation. One study results show that the DHEA level for young adults is three times higher in the morning (8 a.m.) compared with the level in the afternoon (4 p.m.) (Ceresini et al., 2000). Another recent example from Jian et al. shows that diurnal changes and food intake may impact plasma ethanolamide concentrations (Jian et al., 2010). To better understand the variability of plasma ethanolamides in humans, a phase 0 study was conducted. The phase 0 study did not involve administration of any novel or marketed pharmaceutical agent. It was a single-center study that simulated typical single ascending dose and multiple ascending dose studies in population, diet, and activity restrictions. Plasma samples were collected for measurement of basal ethanolamide levels. The observations on causal variations of plasma ethanolamide levels provide valuable information that could be used to facilitate the design of a phase I study where ethanolamide levels could serve as a biomarker of drug treatment. In another study, a variety of biomarkers for lung cancer, such as squamous cell carcinoma antigen (SCC), carcinoembryonic antigen (CEA), and cytokeratin 19 fragments (CYFRA 21-1), were examined in patients' blood samples. No circadian difference was found when a blood sample was withdrawn in the morning, in the afternoon, or in the evening from the same patient (Kahn et al., 2015).

When to establish the normal baseline level of biomarkers has been a debated issue, especially for biomarkers measured a short time before the onset of disease. If the time of sample collection is within the period of the onset of the disease, but before it was clinically manifested, there is a chance that some of the biological parameters measured are the result of the disease, but not of predictive value for the disease. Samples collected a long time before the onset of the disease may be more informative and better associated with the cause of the disease (Hunter, 1997). One example is the correlation of leukemia with the detection of cytogenetic damage in peripheral lymphocytes. If the collection of lymphocytes took place in a relatively short time before the onset of leukemia, it may not be clear whether the cytogenetic damage preceded the disease or if it is one of the resulting abnormalities caused by leukemia (Smith and Zhang, 1998). For many proteomics and inflammatory biomarkers, data are too limited to make specific recommendations about optimal timing of sample collection. Therefore, continued diligence to generate and

track all the variables when collecting biomarker samples becomes very important for the biomarker community.

To elucidate the effects of drug treatment on certain biomarkers in preclinical or clinical studies, consideration has to be given to the sample collection schedule based on the inter- and intra- subject viability and expected change in the biomarker level. If the change is expected to be significant, inter-subject comparison between placebo subjects and the treated subjects should be sufficient. If the change will be small and there is large inter-subject difference in the baseline levels, an intra-subject comparison with a crossover study design can maximize the possibility to detect the effect of drug treatment. If the time frame and magnitude of the change is not known, serial bleeding with short intervals rather than sparse collection can also increase the chance of reliably detecting any changes. Once the trend is better understood, less frequent collection schedule at key time points can be adopted for the subsequent studies. Phase 0 study that elucidates the diurnal changes and food effects of the biomarkers can also help understand the natural variations and facilitate design of the studies.

## 8.3 Matrix Type

Establishing the preferred matrix for analysis is a first key step prior to implementing a biomarker assay. Common matrices include blood and blood-derived fluids, urine, and tissues.

### 8.3.1 Serum or Plasma

Blood and blood-derived fluids are among the most preferred biological specimens owing to the fact that differences in blood chemistry and composition reflect changes in an individual's health status (Lim et al., 2011). Plasma is the noncellular component of blood prepared by centrifugation with the use of anticoagulants, such as ethylenediaminetetraacetic acid (EDTA), sodium citrate, or heparin. Serum is the fluid product of coagulated blood that is devoid of clotting factors and cells. The choice of serum or plasma impacts assay and test outcomes (Luque-Garcia and Neubert, 2007). The effect of these matrix selection choices has been extensively researched in the case of assays and tests involving hormones, antibodies, proteins, enzymes, and amino acids, among other analytes (Cherpes et al., 2003; Chuang et al., 1998; Ferre et al., 2005; Lam et al., 2004; Miles et al., 2004; Rai et al., 2005; Tammen et al., 2005; Tworoger and Hankinson, 2006). For example, plasma is preferred over serum for amino acid analysis. When blood clots, amino acids may be released from or metabolized by erythrocytes, leukocytes, or platelets and are then dispersed into

the serum. Thus, serum has relatively higher and more variable concentrations of most free amino acids than plasma (Scriver et al., 1971). Another example for the differences between serum and heparinized plasma is related to the consumption of fibrinogen and the lysis of cellular elements during the process of clotting. Thus, potassium values are higher in serum than in heparinized plasma, while in contrast total protein values are higher in plasma than in the serum (Berg et al., 1988; Doumas et al., 1989). Since serum sample does not involve any anticoagulant, it is considered to be cleaner or has less interference than a plasma sample for many ligand binding assays (LBA). For the LC-MS assay, an anticoagulant interference is not much of a concern. On the other hand, plasma sample has been found to be a more reproducible sample because blood is still considered as an active sample and EDTA or other protease inhibitors added during plasma collection can control the activity. Thus, the sample choice and preparation considerations are essential factors that have to be planned out carefully at a very early stage of biomarker discovery in order to ensure for the successful transition of a newly discovered marker to clinical setting, especially in the case of blood-based biomarkers (Jambunathan and Galande, 2014).

### 8.3.2 Urine

Protein in urine originates from inefficiencies of plasma glomerular filtration, excretion from epithelial cells in the urinary tract, sloughing of epithelial cells and casts, and formation of urinary exosomes. Changes in urine protein components and concentrations may reflect on dysfunction of cells within the urinary tract itself, while other diseases may be detectable via the transmission of protein fragments from blood into the urine. Urine is a complex and diverse source of candidate protein biomarkers; more than 1500 proteins were identified in a study employing liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) peptide sequencing for identification of urinary proteins separated by SDS-PAGE and reversed-phase liquid chromatography prior to tryptic digestion (Adachi et al., 2006). Another study using capillary electrophoresis coupled to mass spectrometry resulted in identification of as many as 4094 peptides in unfractionated and undigested samples (Coon et al., 2008).

Several properties make urine a favorable source for proteomic biomarkers. First, urine can be obtained in large quantities using noninvasive procedures, and trained personnel are not required for its collection. Due to the noninvasive nature of the procedures, patients are generally willing to donate samples on consecutive occasions; hence, urine is perfectly suited for noninvasive

monitoring of disease progression or response to a therapy. Second, urine contains proteins and peptides of low molecular weight, which can be analyzed by avoiding excessive manipulation, even without tryptic digestion since the digestion produces undesired missed cleavages, increasing sample complexity and variability. Third, urine is a highly stable body fluid, probably because it is stored for hours in the bladder; hence proteolytic degradation by endogenous proteases is essentially complete at the time of voiding. The urinary proteome does not change fundamentally if urine is stored for several years at -20°C (Theodorescu et al., 2006) and the urine protein profile does not change significantly when stored for 3 days at 4°C or after repeated freeze/thaw cycles (Mischak et al., 2013; Schaub et al., 2004).

Potential urinary protein biomarkers have been reported for several types of cancer. Assays for bladder tumor-associated antigen (BTA) (Ellis et al., 1997) and nuclear matrix protein (NMP22) (Grossman et al., 2005, 2006; Saad et al., 2002) in urine have already been approved by the FDA for bladder cancer screening. Urinary cathepsin D has been reported as a potential prognostic biomarker for renal cell carcinoma, correlating well with survival in studies utilizing mass spectrometry and immunoassays (Vasudev et al., 2009). Many candidate biomarkers for diagnosis, prognosis, and selection of treatment are in the development pipeline (Anderson et al., 2009b; Corso et al., 2003; Feldman et al., 2009; Hazzaa et al., 2010; Kageyama et al., 2004; Kaya et al., 2005; Kreunin et al., 2007; Sirohi et al., 2001). Biomarker panels from urine are also under investigation to improve positive predictive value with a goal of utilizing molecular information to tailor treatment regimens to each patient (Koomen et al., 2008). There is also growing evidence that miRNAs, which are small noncoding RNAs (ncRNAs) made up of 19–23 nucleotides, play regulatory roles in gene expression. In addition to other diseases, miRNAs have been found to be dysregulated in urologic diseases, such as malignancies of the prostate, bladder, and kidney (Catto et al., 2011).

### 8.3.3 Tissue

There is an increasing need for quantitative tissue analysis of protein biomarkers in the discovery and development of new medicines. Biomarkers have been historically considered analytes measured in the blood/serum to determine systemic events. Identification of biomarkers directly from tissues of interest or sites of disease (e.g., tumor) can provide a more proximal assessment and therefore may have more value than circulating biomarkers as they are accompanied by contextual information. The tissue samples provide the opportunity to detect spatially related information and the possibility

to assess the interaction of different cell types and markers (Carvajal-Hausdorf et al., 2015). Potential biomarkers in tissue are identified for breast, bladder, and kidney cancers (Chung et al., 2013; Di Napoli and Signoretti, 2009; Feldman et al., 2009). For example, a cathepsin protease inhibitor, cystatin B (stefin B), was evaluated in bladder cancer tissue, and the cystatin B level was increased in cancer tissue compared with normal bladder. The cystatin B modulation was most distinct in high-grade transitional cell carcinoma tissues (Feldman et al., 2009).

## 8.4 Collection Methods

### 8.4.1 Plasma Sample Collection

Blood specimens are extensively utilized traditionally as a sample resource containing a variety of disease biomarkers. Blood-derived plasma and serum samples are readily available for small molecules and proteomic analysis. The general procedure of plasma sample collection for small molecule and proteomic samples is very similar. An example of the collection procedure is listed below:

- a) Collect blood sample using a Vacutainer® collection tube containing an anticoagulant, such as EDTA, heparin, or citrate.
- b) After obtaining the blood sample, mix collection tube thoroughly by slowly inverting the collection tube several times.
- c) Place the collection tube in an ice/water bath and maintain chilled until centrifugation.
- d) Within 60 min of collection, process collection tubes in a refrigerated centrifuge set at approximately 2000×g for 15 min.
- e) Transfer plasma aliquots to a polypropylene screw-cap tube and store it in a freezer set to maintain a temperature of -70°C until shipped for analysis.

In this sample collection procedure, factors that can affect the stability of potential biomarkers include anticoagulants, stabilizing agents (would be added in step (e)), temperature and timing before initial processing, and endogenous degrading properties.

#### 8.4.1.1 Anticoagulants

Traditionally, anticoagulants are used to prevent blood coagulation of samples for future bioanalytical analysis. The choice of different anticoagulants could impact the measurement of the biomarker samples.

Heparin salts are extensively used as anticoagulants in blood collection tubes. The standard commercial grade heparin is a mixed material with a molecular mass ranging from 3 to 30 kDa. Heparin complexes induce a

conformational change of antithrombin III to accelerate the inhibition of thrombin and factor Xa (Capila and Linhardt, 2002), which prevents thrombin activation and the generation of fibrin from fibrinogen. Various salts of heparin, like lithium, sodium, and ammonium, have been used as anticoagulants for blood sample collection. The amount of heparin used in the sample collection tube ranges from 10 to 30 US Pharmacopeia (USP) unit/mL of blood. Heparinized protein samples appear to be relatively stable compared with the citrate and EDTA samples (Dammann et al., 2006). Since heparin binds electrolytes and changes the concentration of bound and free ions, manufacturers have created electrolyte-balanced formulations. However, heparin can interfere with a variety of clinical assays. For example, proteomic studies show that heparinized plasma may cause nonspecific protein binding, which influences the chromatographic separation and mass spectrometer analysis of peptides (Tammen et al., 2004).

Potassium and sodium salts of EDTA have been used for blood specimen collection. The solubility of potassium salts is better than sodium salts of EDTA. EDTA acts as an anticoagulant by chelating calcium ions, which is undesirable if that sample is to be used for a calcium assay. EDTA-treated blood is only slightly less stable than citrate or heparin treatment, but over longer periods of time (4 h vs. 24 h), the sample becomes more unstable as the time increases before centrifugation (Banks et al., 2005). The EDTA-treated blood should be processed as soon as possible to avoid the degradation. Because the activity of many proteases requires metals, the chelating action of EDTA may help prevent protein hydrolysis compared with the untreated serum samples. Plasma protein profiles obtained by EDTA treatment were most divergent from those obtained by citrate or heparin treatment (Hsieh et al., 2006). This may be due to the fact that EDTA causes platelet clumping and aggregation, which might change the protein contents of plasma (White, 2000).

Trisodium citrate solution is preferred for coagulation testing, such as prothrombin time and activated partial thromboplastin time, because it can inhibit both aspartate aminotransferase and alkaline phosphatase by the chelation of cations (Narayanan, 2000). However, this liquid form in the collection tubes dilutes the plasma and may cause issues for the bioanalytical measurement. Sodium citrate, which inhibits platelet activation, is also used to measure plasma levels of platelet-derived components (Narayanan, 2000).

Sample collection for vitamin C is one example that highlights the significant impact that different anticoagulants can have on the quantitation of a biomarker. Vitamin C is present in plasma in two main forms: the reduced form ascorbic acid (AA) and the oxidized form

dehydroascorbic acid (DHAA). The ratio between DHAA and AA may serve as an important plasma biomarker of oxidative stress. AA is the most abundant form in plasma and it is sensitive to oxidation and degradation during blood sampling. Significant differences in baseline AA level in plasma containing different anticoagulants have been observed (Karlsen et al., 2007). The highest AA levels were observed in heparin- and EDTA-treated plasma compared with the citrate-treated plasma. Another example for selecting the right anticoagulant is related to its impact on proteases following blood sampling. Proteases are abundant multifunctional enzymes that play key roles in numerous and diverse physiological processes. Many proteases are a major focus of attention for the pharmaceutical industry as potential drug targets and diagnostic or prognostic biomarkers. Jambunathan et al. used a synthetic combinatorial peptide library of internally quenched fluorogenic peptide probes to map the proteolytic activity profile of the plasma samples that were isolated by different anticoagulants (Jambunathan and Galande, 2014). Their results showed that plasma EDTA is less proteolytically active than the plasma citrate and plasma heparin samples. The difference is believed to be caused by EDTA, which is a known chelator of divalent cations and inhibits various members of the metalloprotease class as well as proteases of other classes that require divalent cations to be active.

#### 8.4.1.2 Stabilizing Agents

Many potential biomarkers are labile and need to be preserved using stabilizing agents. For example, sodium fluoride and lithium iodoacetate inhibit the glycolytic enzyme enolase and glyceraldehyde-3-phosphate dehydrogenase. Inhibition of the glycolytic enzymes limits the ex vivo consumption of glucose by cells in a collected blood specimen (Chan et al., 1989). In the absence of glycolytic inhibitors, a decrease in glucose levels of as much as 24% can occur in 1 h after blood collection in neonates (Meites and Sanielbanrey, 1979). Even with the use of the glycolytic inhibitor, the glucose level in the blood samples is still metabolized at approximately 5–7% per hour at room temperature because upstream enzymes continue to convert it to glucose-6-phosphate (Sidebottom et al., 1982). Hence, complete inhibition of glycolysis in fluoride-containing tubes can take up to 3–4 h. Once the glycolytic inhibitors become fully effective after the initial 3 h period, glucose levels remain stable for at least 3 days (Chan et al., 1989; Li et al., 2013; Sidebottom et al., 1982). The reason that fluoride and iodoacetate can take approximately 3 h to stabilize glucose levels is that it does not act on the first enzyme in the glycolytic pathway, which is hexokinase. Instead, fluoride acts on enolase, which is the eighth enzyme in the glycolytic

pathway, while iodoacetate acts on glyceraldehyde-3-phosphate dehydrogenase, which is the fifth enzyme in the pathway in which glucose is metabolized. The drawback for using sodium fluoride is that it may be unsuitable for enzymatic immunoassays due to its enzyme inhibitory activity and it may also interfere with electrolyte measurements by depleting the ATP content of the red blood cell, which causes an efflux of potassium out of the cell (Li et al., 2013). Iodoacetate can also interfere with measurement of glucose, sodium, potassium, chloride, and lactate dehydrogenase and can cause hemolysis (Li et al., 2013). Analytes that are susceptible to oxidative damage, such as catecholamines, require stabilization by a mixture of AA, EDTA, and egtazic acid (Narayanan, 1998), and the measurement of folate in blood requires stabilization by a mixture of AA and EDTA once the blood sample is collected (Kerkay et al., 1977).

Human blood contains enzymes and proteases, which alter proteins in vivo and ex vivo. Many degradation products could be generated ex vivo during blood collection and sample storage. There are many naturally occurring proteases that are characterized by the nature of their active center. These proteases include serine, cysteine, aspartic, and matrix metalloproteases. Serine proteases contain a serine and histidine in their active center, whereas cysteine proteases contain a cysteine; aspartic proteases contain an aspartic acid group and metalloproteases have a cation like  $Zn^{2+}$ ,  $Ca^{2+}$ , or  $Mn^{2+}$ . Many of the proteases in plasma are released from activated, dying, or lysed neutrophils (Chertov et al., 2000; Fauruschou and Borregaard, 2003; Weiss, 1989) or mononuclear phagocytes (Robbie and Libby, 2001). Plasma also contains an abundance of protease inhibitors whose function is to arrest the activity of proteolytic enzymes. These protease inhibitors include  $\alpha_1$ -protease inhibitor, tissue inhibitor of metalloprotease (TIMP),  $\alpha_2$ -macroglobulin, and plasminogen activator inhibitor-1. However, because of the large quantities and variety of proteases that can be released into the blood by neutrophils and mononuclear phagocytes such as neutrophil elastase, matrix metalloprotease (MMP)-2, and MMP-9 (Boyanton and Blick, 2002; Clark et al., 2003), protease inhibitors normally found in plasma may not be completely effective. It has been found that using a protease inhibitor in combination with an anticoagulant can substantially improve the stability of some biomarkers. For example, aprotinin (Trasylol), a protease inhibitor, can be used in combination with EDTA or heparin to stabilize labile polypeptide hormones, such as glucagon, corticotropin, enzyme renin, and gastrointestinal hormones, such as  $\beta$ -endorphin, secretin, neuropeptides, and gut glucagon (Narayanan, 2000). In addition, using specific cocktails of protease inhibitors, such as BD P800 blood collection kit (BD, Franklin Lakes, NJ) that contains dipeptidyl

peptidase-4 inhibitor and other peptidase inhibitor(s), can preserve glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), oxyntomodulin (OXM), and glucagon plasma samples with half-lives >96, 96, 72, and 45 h at room temperature (Yi et al., 2015).

Acidification is another way to stabilize potential biomarkers. For example, nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) is a ubiquitous coenzyme existing in living cells that is vital not only for energy transduction but also as a key component of signaling pathways. The monitoring of basal  $\text{NAD}^+$  levels and changes in its concentration *in vivo* could help understand the relationship between tumor growth and de novo and salvage pathways of  $\text{NAD}^+$  formation.  $\text{NAD}^+$  levels in the blood have been shown to decrease 60% within 1 h after one freeze/thaw cycle (Liang et al., 2014). The study results showed that  $\text{NAD}^+$  levels can be stabilized by adding 0.5 N perchloric acid solution into the blood sample after sample collection. In addition, the study results showed that perchloric acid added before thawing of the frozen blood sample can also stabilize the  $\text{NAD}^+$  level. The recommended procedure for  $\text{NAD}^+$  blood sample preparation was to add perchloric acid immediately after the blood sample was taken out of the freezer. This procedure has the added benefit of avoiding the use of a strong acid in the animal and clinical facilities (Liang et al., 2014; Liu et al., 2016). Another example of the utility for acidification is for the determination of AA. Degradation of AA in plasma at room temperature and at refrigerated condition (4°C) is about 80 and 30%, respectively in 24 h. If the plasma sample is acidified by equal volume of 10% metaphosphoric acid (MPA), the degradation of AA slows down significantly at room temperature and at 4°C (Karlsen et al., 2007).

#### 8.4.1.3 Temperature and Timing

##### before Initial Processing

Temperature can affect the biomarker stability in different stages: during the time between sample collection and sample processing and during short- and long-term storage. For most of the cases, the biomarker samples are separated into different components (plasma, cells) immediately after collection, and each component is kept at the appropriate temperature. In general, isolated DNA sample is stored at 4°C for several weeks, at -20°C for several months, and at -80°C for several years (Steinberg et al., 1997). Isolated RNA must be stored at -80°C. Serum and plasma contain a large number of soluble molecules and most require very low temperature to remain intact (-80°C). Immunoglobulins in plasma are considered more stable, even when stored at room temperature for up to few days.

Temperature control during the time between sample collection and sample processing is essential, especially when it takes several hours. The appropriate temperature depends on the biomarkers of interest, and it needs to take into account the temperature required for stabilizing of each biomarker during the study design. If a very labile biochemical biomarker is the main focus of the study (e.g. cytokines) and the samples are not going to be analyzed immediately, they should be frozen at -80°C, and repeated freeze/thaw cycles must be avoided. If the experiment requires maintaining the cell viability for longer time, then the sample may be kept at room temperature for up to 48 h, but this is incompatible with preservation of labile biomarkers of protein nature (e.g. cytokines), antioxidants (AA, uric acid,  $\alpha$ -tocopherol), and others, such as folate and vitamin B12. Low temperature (4°C) storage is often a good compromise between the two extremes of freezing or room temperature: cells can remain viable (reduced viability compared to room temperature), and it also protects, at least to some extent, against enzymatic degradation of labile biomarkers.

The allowable time between collection and processing of biological samples depends on the components of interest and their stability. Ideally, when separation of plasma or serum from cells is needed, blood should be processed within 1 h of collection to obtain plasma or serum. For many biomarkers the time between collection and processing affects their stability. One example is the diminution of folate stability over time (at room temperature). Delays between collection and processing will affect the estimate of folate levels (Drammeh et al., 2008). Similarly, AA is rapidly degraded in blood and plasma. Sample collection should be followed by immediate centrifugation and plasma acidification (Karlsen et al., 2007). For cytokine analysis, cells must be separated from serum immediately after blood collection, again because delays between collection and processing will affect the results (House, 2001). These considerations would determine when and how the collection and processing take place. For example, if the physical distance between the collection and processing facilities involves mailing or transportation delays, unstable biomarkers should be excluded. Alternatively, minimal initial steps should be conducted before sample transfer to assure its integrity.

#### 8.4.1.4 Endogenous Degradation

Enzymatic degradation affects many biochemical biomarkers. Proteins are sensitive to degradation by proteases, particularly if cell integrity has been compromised. Protein integrity can be protected by addition of protease inhibitors, such as  $\alpha_2$ -macroglobulin, aprotinin, leupeptin, pepstatin, chymostatin, phenylmethylsulfonyl fluoride, benzamidine, and diisopropyl fluorophosphates,

to the sample immediately after collection. One must know that protease inhibitors are toxic to live cells and therefore should be avoided if cell viability is desired. Furthermore, all steps during protein handling should take place on wet ice bath. RNA is also particularly sensitive to degradation by abundant and ubiquitous ribonucleases (RNase). RNA samples should be handled with RNase-free tubes and the addition of commercially available RNase inhibitors. Unlike proteins and small molecules, RNA is not protected at low temperatures. In contrast, DNA is the most stable component in biological samples, including blood, exfoliated cells, and other tissues. There are reports showing that DNA from exfoliated cell specimens was stable for up to a week at room temperature. In fact, exposure to 37°C for a week also does not affect the DNA yield (Lum and Le Marchand, 1998).

#### **8.4.2 Urine Sample Collection**

For proteomic and protein biomarkers, urine samples demonstrate a high degree of variability in volume, protein concentration, pH, and variability in urine components. Several urine sample collection protocols, including 24-h urine, first morning urine, and spot urine collection, are currently used in clinical studies. Twenty-four hour collection can be awkward for patients and may lead to degradation and contamination of urine protein, particularly via lysis of suspended cells, because the samples are stored at 4°C and often transported under ambient conditions. Single sample collection is more convenient for patients, is more easily standardized, and enables quicker processing and storage of samples. Among single sample collections, first morning urine collection displays somewhat higher variability, but such collection minimizes the amount of time spent in the bladder, where increased proteolysis may occur. Additionally, random spot collection facilitates coordination between patients, clinicians, and researchers.

A midstream, clean catch urine collection minimizes problems with bacterial contamination. In fact, studies have shown difference between the first void and mid-stream urine samples, indicating that the collection method can be important (Schaub et al., 2004). Similar to serum and plasma samples, protease inhibitors are often added to urine samples to preserve intact protein forms (Berg et al., 1988; Doumas et al., 1989). To obtain protein biomarkers from urine, it is found that centrifugation performed within 20–30 min of collection minimizes contamination of the urine due to lysis of suspended cells (Thongboonkerd, 2007). Ultrafiltration has been reported to be the best method for concentration and cleanup of peptide and protein components from urine

and facilitates collection of lower molecular weight analytes and buffer exchange for downstream processing (Nabi et al., 2005; Pieper et al., 2004; Ru et al., 2006; Tantipaiboonwong et al., 2005). Alternatively, protein precipitation using organic solvents (Oh et al., 2004; Soldi et al., 2005; Sun et al., 2005), dialysis (Castagna et al., 2005; Oh et al., 2004; Smith et al., 2005), lyophilization (Castagna et al., 2005; Oh et al., 2004; Thongboonkerd et al., 2006), and ultracentrifugation (Thongboonkerd et al., 2006) may be used. miRNA has demonstrated good stability compared with messenger RNA (mRNA) and other cellular RNAs even though urine contains abundant nucleases (Juan et al., 2010). Some reports suggest that the miRNA stability is attributed to nuclease resistance based on the smaller nucleic acid size (Hanke et al., 2010) and/or microvesicular containment (Schwarzenbach et al., 2011).

For small molecule biomarkers, analyte loss due to nonspecific binding, especially container surface adsorption, is not uncommon in the quantitative analysis of urine samples (Chen et al., 2009; Groff et al., 2006; Li et al., 2010; Xu et al., 2005b). Urine does not normally contain much protein or lipid compared to blood, plasma, or serum samples. The lack of protein and lipid in urine samples can be associated with the issue of non-specific binding of small molecule biomarkers during urine sample analysis. Although the addition of control human plasma or bovine serum albumin into the urine samples can resolve the issue, their effect may vary significantly depending on the protein-binding properties of different analytes. Other surfactants, such as Tween-20 (Anderson et al., 2009a; Xu et al., 2005a), Tween-80 (Chen et al., 2009), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Chen et al., 2009; Li et al., 2010), sodium dodecylbenzenesulfonate (SDBS) (Chen et al., 2009; Silvester and Zang, 2012), and  $\beta$ -cyclodextrin (Li et al., 2010) were used to effectively improve the recovery of proteins and small molecules in urine by reducing nonspecific binding. Unfortunately, the use of surfactant in urine samples presents different challenges. A relatively high concentration of surfactant in the sample introduces a further, and not insignificant, interference in an already complex matrix. For LC-MS/MS-based assays, this interference may manifest itself as suppression (or enhancement) of the analyte response (Xu et al., 2005a). Therefore, it is important that the surfactant is characterized appropriately prior to the analysis of the urine samples. The selection of a suitable surfactant may obviate the need for any specific sample preparation procedure for the removal of the surfactant added. In addition, variable pH or ionic strength of urine can lead to inconsistent extraction recoveries and nonreproducible

analyte LC–MS/MS response between runs. This issue can be addressed by adding an acid (such as acetic acid, hydrochloric acid, or citric acid) during sample collection to control the pH of urine samples.

#### 8.4.3 Tissue Sample Collection

Tissue sample acquisition, processing, and storage procedures can affect the quality of the specimen collected. There are two different variable time periods that can affect the biomarker stability during the tissue procurement: (a) post-excision delay time and (b) processing delay time. The post-excision delay time is the variable time frame between specimen excision and the point at which the specimen is placed in a stabilized state, such as immersed in fixative or snap-frozen in liquid nitrogen. During the post-excision time frame, the tissue may reside at room temperature, or it may be refrigerated, either in a closed or open container. The second variable time period is the processing delay time. Common variables associated with processing delay time are the permeation rate of the fixative through the tissue and the length of time to freeze the specimen. Both these time periods should be minimized to prevent biomarker degradation. For example, the difference of temperature can significantly change the RNA expression levels of several genes in mouse liver tissue at 25 or 37°C. When mouse liver tissue samples were incubated at 37°C for 4 h, the mRNA levels dropped to 10% of the levels measured at 25°C (Almeida et al., 2004). Similar to plasma and serum samples, transporting the tissue in a sterile and RNase-free container on wet ice and promptly freezing an aliquot appear to be the best way to preserve RNA biomarker integrity. Protein phosphorylation for the tissue sample can be avoided by rapid freezing (<5 min) in liquid nitrogen. Walker et al. showed an increase in troponin I phosphorylation and a decrease in troponin T and myosin light chain 2 phosphorylation in tissues that were kept in cardioplegia when compared with samples that were immediately frozen in liquid nitrogen (Walker et al., 2011).

### 8.5 Sample Storage Stability

#### 8.5.1 Storage of Blood-Derived Fluids and Urine Samples

Depending on which biomarker is the focus of interest, the temperature for short- and long-term storage may vary. Typically, collected blood samples are processed to obtain plasma or serum samples as soon as possible to avoid any drop in biomolecule yield and to minimize potential degradation. It has been shown that DNA can

be extracted with acceptable yield and quality from blood samples that are stored at room temperature, 4°C, and at -20°C for a maximum of 1 month (Nederhand et al., 2003). Prolonged storage causes lysis of the erythrocytes, and, with severe hemolysis, it is expected that some of the leukocytes will lyse as well, which will result in loss of DNA during the leukocyte harvesting step in DNA extraction (Nederhand et al., 2003). In this respect, when processing cannot be undertaken immediately, blood samples should be frozen at -80°C to improve DNA yield (Steinberg et al., 2002). While extracted DNA from blood samples is stable at 4°C for several weeks, at -20°C for months, and at -80°C for years (Steinberg et al., 1997), RNA is more labile and degrades quickly at temperatures higher than -80°C. It has also been shown that the integrity of miRNA is maintained for years in plasma samples stored at -80°C, highlighting that not all RNA species are equally susceptible to degradation (Grasedieck et al., 2012; Juan et al., 2010). Serum and plasma contain a large number of soluble molecules, including many proteins that have varied stability. Immunoglobulins in plasma are considered stable, even at room temperature for few days, whereas other proteins are labile and remain stable at 4°C only up to 24 h (Hsieh et al., 2006), therefore requiring very low temperature to stay intact for long-term storage (-80°C) (Holland et al., 2003). No degradation of protein and other molecules was seen in plasma samples stored at -80°C or liquid nitrogen for up to 6 years (Elliott and Peakman, 2008; Lewis et al., 2001). A recent study investigated the stability of multiple biomarker classes in a biobank (Zander et al., 2014). Results showed no substantial changes when the samples were stored at -80°C or below -130°C. However, substantial changes for some biomarkers were found when the samples were stored at 4°C and/or -20°C.

For urine samples, because the collection protocol is usually different than the one used for blood sample collection and most of the urine sample will not be able to be processed immediately after the collection, the short-term storage at room temperature and 4°C needs to be investigated. Several biomarkers, such as neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), and interleukin 18 (IL-18), were used for the detection and prognostication of acute kidney injury. Their storage stability was investigated, and results showed that the urine samples should not be stored at 25°C for even short (24 h) periods because it leads to significant decrease in concentrations of NGAL, KIM-1, and IL-18 (Schuh et al., 2016). These samples could be stored at 4°C up to 24 h if the samples cannot be processed immediately. Common practice is to store the plasma, serum, and urine samples at -80°C for long-term storage.

### 8.5.2 Storage of Tissue Samples

In the recent years, ultralow temperature ( $-80$  to  $-150^{\circ}\text{C}$ ) became standard for long-term storage as it became clear that tissue degradation did occur at  $-20^{\circ}\text{C}$ . It has been suggested that storage at below  $-137^{\circ}\text{C}$  may be the optimal temperature as  $-137^{\circ}\text{C}$  is the glass transition temperature ( $T_g$ ) of water, below which biochemical activity that might degrade intracellular contents are thought to be inert. With liquid nitrogen, vapor phase storage ( $-150^{\circ}\text{C}$ ) is generally preferred over liquid phase ( $-196^{\circ}\text{C}$ ) because of the risk of contamination by errant floating tissue fragments in liquid nitrogen. Effects of a range of storage temperatures on tissue sample stability have been studied. Study results showed that DNA yield and integrity remains unchanged for the tumor tissue as long as for 7 years at  $-80^{\circ}\text{C}$  (Chu et al., 2002). Some RNA brain autopsy tissue samples can be stored at  $-70^{\circ}\text{C}$  for 15 years (Yasojima et al., 2001). For long-term storage, specimens should be stored at least at  $-80^{\circ}\text{C}$ .

### 8.5.3 Freeze/Thaw Effect

Stability of DNA, RNA, protein, and small molecule biomarker in blood, urine, and tissue samples are affected by freeze/thaw cycles. Study results showed that a single freeze/thaw cycle can decrease the yield of DNA by 25% in blood samples (Ross et al., 1990) and repeated freeze/thaw cycles can lead to significant changes of serum and plasma proteomes (Baumann et al., 2005). Several studies have found that many repeated freeze/thaw cycles lead to significant decreases in RNA integrity particularly for autopsy brain tissue (Atz et al., 2007; Ma et al., 2012; Sherwood et al., 2011). Vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP)-7 levels in plasma and serum samples significantly changed with the number of freeze/thaw cycles (Lee et al., 2015). NAD<sup>+</sup> levels in blood were also found decreasing 60%

within 1 h after one freeze/thaw cycle (Liang et al., 2014). In contrast, one study result showed that repeated freezing at  $-80^{\circ}\text{C}$  and thawing on ice for up to 10 times did not present any obvious changes in low molecular weight serum and plasma proteomes (Hsieh et al., 2006). A couple of studies found that repeated freezing and thawing of ovarian and brain tumor samples caused no alterations in RNA quality and protein expression (Ericsson et al., 2007; Jochumsen et al., 2007). The mixed finding in these studies indicated the importance to evaluate the freeze/thaw parameters for the samples. In general, it is believed that freezing/thawing of blood, urine, and tissue samples should be avoided or minimized. If freeze/thaw cannot be avoided, placement of samples on dry ice or wet ice during sample process should be considered.

## 8.6 Summary

We have presented a series of considerations related to sample collection and storage of biological specimens for biomarker quantitation. The advances in examination of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention can only be taken advantage of if sample quality is assured for already available and future biomarkers. Proper handling of biological samples from the time of collection to the analysis protects the quality of the specimens and the validity of the results. Tables 8.1, 8.2, and 8.3 summarize the important considerations for the biomarker sample collection. Actions to be taken include the identification of an appropriate specimen to be used for the biomarker analysis, determination of the timing of collection, and examination of the biomarker stability. Careful understanding of the effects of each preanalytical variable on the biomarker data will help to ensure the quality of the biomarker results.

**Table 8.1** Preanalytical considerations for blood samples.

Preanalytical phase	Variable	Considerations
Sample type	Serum or plasma	Interference of anticoagulants, protein profiles, sample variability
Treatment	Additives, such as anticoagulants, protease inhibitors, acidification	Intended inhibition of coagulation, protection from biomarker degradation, or modification ex vivo
	Tube type, such as RNase-free tube	Improve stability of RNA
Sample processing	Processing time and temperature	Biomarker stability, hemolysis
	Centrifugation time, force, and temperature	Biomarker stability, hemolysis
Sample storage	Temperature during short- and long-term storage	Biomarker stability
	Freeze/thaw cycles	Biomarker stability

**Table 8.2** Preanalytical considerations for urine samples.

Preanalytical phase	Variable	Considerations
Treatment	Additives, such as protease inhibitors, acidification, and surfactants	Biomarker stability, nonspecific binding
Timed collections	Spot (random), first morning, or long-term (24 h) collection	Variability in biomarker excretion
Sample processing	Whole urine or centrifuged (supernatant)	Potential for cellular contamination in whole urine
Sample storage	Temperature during short- and long-term storage Freeze/thaw cycles	Biomarker stability Biomarker stability

**Table 8.3** Preanalytical considerations for tissue samples.

Preanalytical phase	Variable	Considerations
Sample processing	Post excision delay and processing delay time	Biomarker stability
Sample storage	Temperature during short- and long-term storage Freeze/thaw cycles	Biomarker stability Biomarker stability

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**9****Nonspecific Binding in LC–MS Bioanalysis***Aimin Tan<sup>1</sup> and John C. Fanaras<sup>2</sup>*<sup>1</sup> Bioanalytical Laboratory, Nucro-Technics, Scarborough, Ontario, Canada<sup>2</sup> Nucro-Technics, Scarborough, Ontario, Canada**9.1 Introduction**

Nonspecific binding (NSB, also known as nonspecific adsorption or aspecific adsorption or binding) refers to the unintentional or unexpected adsorption or binding of compounds of interest (e.g., analyte and/or internal standard (IS)) to the receptor of noninterest, such as adsorption of analyte on container surfaces or pipette tips. Although this is termed as nonspecific, the binding could sometimes be quite specific in nature (such as in the cases of tetracyclines and phosphorylated compounds complexed with the residual metal ions in an LC column or metal parts of an LC–MS system) (Santos et al., 1996; Tuytten et al., 2006). This undesirable binding, if left uncontrolled, can pose severe challenges for LC–MS bioanalysis, from chromatographic peak tailing, carry-over, low and variable recovery, nonlinearity, and low sensitivity (high detection/quantitation limit) to ultimately imprecise and inaccurate results (Gu et al., 2010; Ji et al., 2010; Warwood et al., 2013; Maes et al., 2014a). Hence, it is very critical to reduce or eliminate the undesirable NSB during LC–MS bioanalysis.

But, first of all, how do we even know NSB exists? How do we evaluate its severity? What are the main causes for NSB? Why is NSB particularly problematic for biomarker quantitation? How can NSB be differentiated from a seemingly similar stability issue? What are the solutions at our disposal to reduce or eliminate NSB? All of these questions will be addressed in this chapter using representative application examples from the LC–MS bioanalysis of both small and large molecules.

**9.2 Identification and Evaluation of NSB****9.2.1 Common Scenarios and Indicators for Potential NSB Issues**

Based on past experiences, NSB should be proactively watched for when dealing with certain types of compounds and matrices. These compounds include peptides, proteins, basic, highly hydrophobic, highly protein-bound compounds, tetracyclines, phosphorylated compounds (e.g., oligonucleotides, phosphorylated peptides, phosphorylated proteins), and compounds containing fluorine or rich in hydroxyl or boronic acid groups (Santos et al., 1996; Tuytten et al., 2006; Hughes et al., 2007; Zhang et al., 2007; van den Broek et al., 2008; Silvester and Zang, 2012; Chen et al., 2014; Maes et al., 2014a). The matrices that are prone to adsorption issues are typically those with very low protein and/or lipid contents, such as urine, cerebrospinal fluid (CSF), dialysate, and saliva (Ji et al., 2010; Li et al., 2010). When the aforementioned compounds and/or matrices are involved, efforts should be made to monitor for NSB.

Losses due to NSB depend on many factors, such as the various surfaces and surface activities a given sample is exposed to and the sequence of the exposure, and the volume, concentration, temperature, and pH of the sample. Since these factors are either difficult to control or not deliberately controlled, “randomality” is usually a hallmark of NSB. Additionally, there is a maximum surface area for a given container (e.g., tube or vial) and

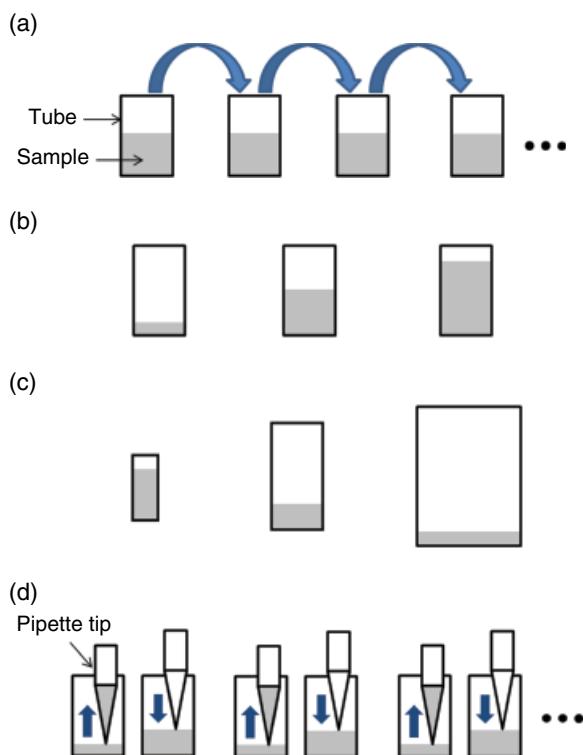
NSB would be saturated at a certain point. Therefore, NSB impact on the quantification of a sample would be determined by the surface and surface activity of the container as well as the volume and concentration of the sample. The lower the volume or the concentration of the sample, and/or the larger and more active the container surface, the higher the impact is (Ji et al., 2010).

For the aforementioned reasons, one or more of the following symptoms usually indicate an NSB issue: (a) an upturned nonlinear calibration (response vs. concentration) curve (Gu et al., 2010; Ji et al., 2010); (b) “stability” or accuracy issues mainly at the low concentration end (Hu and Kamberi, 2009; Xu et al., 2012); (c) low and variable recovery (Oka et al., 1985; Chambers et al., 2013); and (d) systematic errors between calibration standards (CSs) and quality controls (QCs), and poor accuracy and precision (random or “jumpy” results), especially when they are difficult to rationalize or explain. In addition, chromatographic peak tailing and carry-over are also indicators of potential NSB issues (Maes et al., 2014a).

### 9.2.2 Confirmation/Identification and Evaluation of NSB

Once the presence of NSB is suspected, it is important to confirm it and evaluate its severity. Additionally, it is also pivotal to diagnose the place or stage of LC–MS bioanalysis where NSB occurs because different strategies might be needed to mitigate its impact. NSB can occur in neat solutions, which has impact on the preparation/concentrations of working standard solutions, especially at the low concentration end. Common mitigation strategies include the use of organic solvents and/or preparation of CSs using serial dilutions in a control blank matrix. NSB can also occur in biological matrices during sample collection or storage. The loss of analyte due to this nature cannot be compensated for, and the loss could be permanent if a preventive action such as addition of organic solvents or surfactants is not taken. Furthermore, NSB can occur during and/or post the sample extraction, such as losses at the drying-down and reconstitution step or in LC injection vials. Even though the impact of these losses can usually be minimized through the use of a good IS (Tan and Kayode, 2013), for example, stable isotope-labeled IS, the assay sensitivity can be compromised if the losses are significant. Alternative sample extraction procedure and reconstitution solution or use of low-binding LC injection vials might be exemplary solutions to reduce or eliminate NSB losses.

There are some easy and simple approaches to confirm NSB and evaluate its severity (Chen et al., 2009; Ji et al., 2010; Li et al., 2010; Silvester and Zang, 2012). For example, to confirm NSB loss to a type of tube (e.g., glass tube), a homogeneous liquid sample with a known



**Figure 9.1** Different approaches for confirming or evaluating NSB losses to tubes or pipette tips. (a) Multiple sequential transfers among tubes; (b) different sample volumes in tubes of the same size; (c) same sample volume in tubes of different sizes; (d) sequential passing of sample through multiple pipette tips.

concentration can be sequentially transferred from one tube to the next (with a vortex mixing prior to each transfer) multiple times (Figure 9.1a). Then, the sample in the last tube can be analyzed and its concentration compared with the initial or nominal concentration. Alternatively, the homogeneous sample can be aliquoted in different volumes into several tubes of the same size (Figure 9.1b) or be aliquoted in the same volume to a few tubes of different sizes (Figure 9.1c). After vortex mixing, these aliquots can be analyzed and their concentrations compared. If a significant discrepancy (e.g., >15%) in the concentrations is observed, NSB is confirmed.

To confirm NSB loss to a type of pipette tip, a small volume of a homogeneous liquid sample with a known concentration can first be added to a small container. A portion of it can be aspirated into a new pipette tip and then dispensed back to the same container (Figure 9.1d). This process may need to be repeated multiple times with a new tip each time (the number of repetitions will depend on the initial sample volume and the severity of NSB). At the end, the remaining sample in the small container can be analyzed and its concentration compared with the initial or nominal concentration to gauge the severity of NSB.

To evaluate NSB loss to sample collection tubing and/or determine the maximum length of sample collection tubing, a homogeneous liquid sample with a known concentration can be pushed (e.g., by using a syringe) to flow through several pieces of collection tubing with different lengths. Then, the flow-through portions are analyzed, and their concentrations are compared with the initial or nominal concentration. If a significant difference in the concentrations is observed for a given tubing length, NSB is confirmed. Any tubing longer than that length should not be used. Otherwise, a certain amount of sample must be discarded prior to collecting samples for analysis (Gu et al., 2010).

Apart from the aforementioned approaches, the following are also very useful for identifying an NSB issue: (a) deliberately varying the spiking/preparation volumes for CSs and QCs (Ji et al., 2010), (b) serially diluting a high concentration standard solution and a high concentration matrix sample and then comparing their linearity (Gu et al., 2010), and (c) frequently comparing freshly prepared samples with non-freshly prepared samples (Li et al., 2010). Although the aforementioned approaches appear to be different, they all share the same key principle: creating experimental conditions to expose and amplify the impact of NSB so that it will not be missed.

As for carry-over and chromatographic peak tailing, it is relatively easy to spot them. To evaluate carry-over, one or several control blank or neat solution (e.g., mobile phase) samples can be immediately injected after a high concentration sample (Hughes et al., 2007; Maes et al., 2014a). The responses in blank or neat solution samples are compared with the response of the high concentration sample to determine the severity of carry-over. Typically, this should be equal to or less than 20% of the lower limit of quantitation (LLOQ) response. For evaluating the severity of chromatographic peak tailing, a symmetric factor or a tailing factor can be used (Dolan,

2003). It should be pointed out that NSB is not the only factor that causes carry-over or peak tailing. Other factors include the extra dead volume in the flow path. In addition, the surfaces involved (e.g., the rotor and stator of an injector) may be saturated above a certain point. If the concentration of the high concentration sample is too high, the carry-over could be underestimated. Therefore, it is important to test more than one high concentration.

Finally, it should be emphasized that the degree of NSB and its impact on quantification depend on various factors, such as the materials involved, sample matrix, pH, temperature, concentration range, sample volume, and sample processing procedure. Therefore, the aforementioned identification or evaluation tests must be done at conditions relevant/specific to the task at hand. Typically, both low and high concentrations should be evaluated. In addition, for some matrices, like urine, their compositions may change after undergoing freeze and thaw cycles, for example, protein precipitated. Hence, the evaluation test should also include matrices that have gone through freeze and thaw cycles (Ji et al., 2010).

### 9.2.3 NSB versus Stability Issue

As both NSB and stability issues can lead to analyte losses and negative biases for quantification, NSB may sometimes be mistaken for a stability issue (Hu and Kamberi, 2009). Since the solutions to NSB and stability issues are usually different, it is therefore very important to first verify whether the issue at hand is indeed NSB.

Fortunately, notwithstanding their apparent similarity, NSB and stability issues can be different in several aspects, such as concentration dependence, volume dependence, time dependence, material dependence, and reversibility of losses (Table 9.1). Based on these differences, it would be relatively easy to design an

**Table 9.1** Differences between NSB and stability issue.

Property	NSB	Stability issue
Concentration dependence	The lower the concentration of a sample, the higher the impact	Usually similar impact on both low and high concentration samples, but low concentration samples could be affected more in some cases
Volume dependence	The higher the volume of a sample, the lower the impact	Usually independent of sample volume
Time dependence	Binding equilibrium could be reached fairly quickly and no further change once the equilibrium is reached	The longer the duration, the higher the impact
Material dependence	Specific to material type, for example, NSB with plastic tubes, but not with glass tubes	Typically irrespective of tube materials
Reversibility of loss	Could be reversible	Usually not reversible (changed to different chemical entity)

experiment to ascertain the correct issue at hand. For example, low and high QC samples can be aliquoted in the same volume to several plastic and glass tubes of the same size. Some of the aliquots can be analyzed immediately, while others can be analyzed after being stored for different durations (e.g., 1, 5, and 10 h). If a time-dependent decrease is observed for both tube types and both concentration levels, the issue must be a stability one.

### 9.3 Causes for NSB

Although it may manifest itself in different forms or patterns, NSB is usually caused by inadequate solubility and/or relatively stronger interactions (hydrophobic, ionic/electrostatic, van der Waals, hydrogen bonding, etc.) between the compound of interest and various surfaces in comparison with its interactions with the bulk solvent or matrix components. Sometimes, it could even be due to a chemical reaction (e.g., complexion) between the compound of interest and a surface (Santos et al., 1996; Tuytten et al., 2006).

It is very important to realize the competitive nature of NSB. The sheer existence of NSB does not always indicate an issue of inadequate solubility per se. For a homogeneous liquid sample, it may have an NSB issue with one vial type (e.g., plastic vial), but not with another vial type (e.g., glass vial). Evidently, the compound of interest in the liquid sample cannot be both soluble and insoluble. In addition, the impact of NSB is usually more pronounced for low concentration samples, whereas a true solubility issue is normally first shown at high concentrations. Furthermore, it is quite common for a water-soluble compound to still have an adsorption issue in a urine matrix, which is basically aqueous (Schwartz et al., 1997). For the above reasons, NSB is often more of an issue of stronger interaction(s) of the compound of interest with surfaces than with the sample solvent or matrix rather than inadequate solubility as many may believe, though NSB may indeed be due to inadequate solubilization in some cases.

In comparison with small drug molecule bioanalysis, NSB could be much more problematic for the LC-MS bioanalysis of biomarkers for two major reasons. Firstly, the matrices of choice in biomarker bioanalysis are more diverse, including many matrices that have very low protein and/or lipid contents (e.g., urine, CSF, dialysate, saliva). These low protein and low lipid matrices are prone to NSB issues. Secondly, in many cases, the compounds of interest are more complex, like peptides or proteins, which have both positive and negative charge sites as well as both hydrophobic and hydrophilic residues (patches) on the same molecule. In addition, they

may exhibit special configurations due to their large sizes. For these reasons, there is usually more than one type of interactions coexisting. The elimination or reduction of a single type of interaction or a portion of the interactions may not solve an NSB issue. In other words, it could be more challenging to find a solution or solutions to the NSB issue(s) in biomarker bioanalysis.

## 9.4 Overcoming NSB Challenges

### 9.4.1 Solubilization of Compounds

The proper solubilization of the compounds of interest at all stages of sample analysis, such as the preparation of stock and working standard solutions, sample extraction, and reconstitution, is the prerequisite for avoiding or reducing NSB. Special attention should be paid to the proper solubilization in working IS and reconstitution solutions. As the commonly used solid-phase extraction (SPE), liquid–liquid extraction (LLE), and supported liquid extraction (SLE) methods dictate a low percentage of organics in a sample-IS (loading) mixture, many may choose to prepare a working IS solution in an aqueous buffer. A lipophilic IS may not be fully soluble in the aqueous buffer. To match the initial low organic gradient and/or to avoid excessive evaporation of extracted samples, low organic reconstitution solutions are often preferred, which may not be able to solubilize lipophilic analytes and/or ISs well.

To solubilize a typical small molecule compound is relatively straightforward. Depending on its hydrophobicity and chemical form (e.g., free base or salt), water, methanol, acetonitrile, or their mixture, for example, methanol/H<sub>2</sub>O (50/50, v/v), can be used. Sometimes, these solvents may need to be acidified or basified. In the case where the compound is still not well solubilized, a small percentage of dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) (e.g., 10–20%) may be added. In extreme cases, the compound can be directly dissolved in DMSO or DMF.

On the other hand, the solubilization of a peptide or polypeptide (protein) is much more complicated because of the coexistence of both positive and negative charges as well as both hydrophobic and hydrophilic moieties/parts in the same molecule. On top of this, there may be inter- and intramolecular interactions like hydrogen bonding and disulfide formation. Furthermore, the possible combinations of amino acid residues and their sequence are almost endless. Because of these factors, there is no universal solvent for solubilizing all peptides.

Nevertheless, it is still possible to obtain the preliminary solubility characteristics of a peptide or protein by

evaluating its amino acid composition. From this preliminary solubility information, an appropriate starting solvent may be chosen (Maes et al., 2014a; Sigma guidelines). Here is how:

- Count the total number of amino acid residues.
- Calculate the % of hydrophobic residues (A, F, I, L, M, P, V, W, and Y).
- Calculate the % of charged residues (D, E, H, K, R, C-terminal COOH, and N-terminal NH<sub>2</sub>).
- Determine the overall net charge by assigning -1 to each acidic residue (D, E, and C-terminal COOH), +1 to each basic residue (K, R and the N-terminal NH<sub>2</sub>), and +1 to each H residue if the pH is less than 6.
- Estimate the tendency for gel formation by calculating the % of D, E, H, K, N, Q, R, S, T, or Y residues (which may form intramolecular hydrogen bonds).

Once the aforementioned parameters are calculated, the general guidelines presented in Table 9.2 can be followed to choose an appropriate starting solvent. Based on the solubilization result in the starting solvent, a final solvent can be found by “trial and error.”

Take human amyloid  $\beta_{1-40}$  peptide as an example. Its amino acid sequence is DAEFRHDSGY EVHHQKLVFF AEDVGSNKGA IIGLMVGGVV. The overall net charge is -3, so it is an acidic peptide. The percentages of hydrophobic and charged residues are 45 and 35%, respectively. In addition, the tendency for the peptide to form gel is low because the percentage of the residues that may form

intramolecular hydrogen bonding is only 43%. According to the guidelines in Table 9.2, a starting solvent would be an aqueous one with a basic additive and low % organic. This is in agreement with the actual solvent used by Oe et al. (2006) for solubilizing this peptide, namely, 0.1% NH<sub>4</sub>OH in water/acetonitrile (4:1, v/v).

#### 9.4.2 Overview of Measures for Overcoming NSB Challenges

Just as the causes for NSB are diverse and the severity of NSB depends on many various factors, so are their potential solutions. Summarized in Table 9.3 are various measures/approaches that may be used to eliminate NSB or reduce its impact during different stages of LC-MS bioanalysis, for example, standard solution preparation, sample extraction, LC-MS analysis, and sample collection and handling.

Among the various measures, some focus on the compound of interest to increase its “affinity” to the bulk solution or matrix, such as by adding organic solvents, surfactants, proteins, and plasma or even by changing its chemical form (by pH adjustment or derivatization) to those that are less likely to suffer from an NSB issue (Schwartz et al., 1997; Oe et al., 2006; Provencher et al., 2014; Zhao et al., 2015; Vasquez et al., 2015). Others aim to make the surfaces less likely to interact with the compound of interest by blocking binding sites, deactivating surfaces, pretreating surfaces, or changing the environment,

**Table 9.2** General guidelines for solubilizing peptides.

Solvent type	Typical applicable scenarios	Comment	
Aqueous or aqueous with low % organic, for example, 5–20%	With basic additives, for example, 1–5% NH <sub>4</sub> OH and 0.1 M NH <sub>4</sub> HCO <sub>3</sub>  With acidic additives, for example, 0.1–1% TFA and formic or acetic acid	a) <5 residues b) >25% charged residues (D, E, H, K, R, C-terminal COOH, and N-terminal NH <sub>2</sub> ) and <50% hydrophobic residues (A, F, I, L, M, P, V, W, and Y) with negative (acidic peptide) or zero (neutral peptide) net charge  a) <5 residues b) >10% charged residues and <50% hydrophobic residues with positive net charge (basic peptide)	The higher the % of hydrophobic residues, the higher % organic to be used
DMSO, DMF, TFA, acetic acid, formic acid, isopropanol, guanidine HCl, urea		a) >50% hydrophobic residues b) <10% charged residues for basic peptides or <25% charged residues for neutral or acidic peptides c) >75% of D, E, H, K, N, Q, R, S, T, or Y residues (tend to form gel due to extensive intramolecular hydrogen bonding).	Avoid using DMSO for peptides containing C or M. Guanidine HCl or urea is good for peptides that tend to aggregate or gel

Notes: A, alanine (Ala); C, cysteine (Cys); D, aspartic acid (Asp); DMF, dimethylformamide; DMSO, dimethyl sulfoxide; E, glutamic acid (Glu); F, phenylalanine (Phe); H, histidine (His); I, isoleucine (Ile); K, lysine (Lys); L, leucine (Leu); M, methionine (Met); N, asparagine (Asn); P, proline (Pro); Q, glutamine (Gln); R, arginine (Arg); S, serine (Ser); T, threonine (Thr); TFA, trifluoroacetic acid; V, valine (Val); W, tryptophan (Trp); Y, tyrosine (Tyr).

**Table 9.3** Overview of measures for overcoming NSB challenges.

<b>Measures/approaches</b>		<b>Standard solution preparation</b>	<b>Sample extraction</b>	<b>LC-MS analysis (carry-over or peak tailing)</b>	<b>Sample collection and handling</b>
Addition or adjustment	Addition of organic solvents, for example, methanol and acetonitrile	+	+	+	+
	Addition of surfactants, for example, Tween-20, Tween-80, CHAPS, and SDBS	+	+	+	+
	Addition of proteins or phospholipids, for example, BSA, HSA, or plasma	+	+	+	+
	Addition of complexing agents, for example, EDTA, oxalic acid, and citric acid	+	+	+	+
	pH adjustment	+	+	+	+
	Ionic strength adjustment	+	+	+	+
Procedure	Using matrix dilution, instead of spiking standard solution, to prepare CSs and QCs	+	+		
	Maintaining/using high volume and/or high concentration; increasing sample volume	+	+	+	+
	Reducing transfer/handling steps	+	+		+
	Coating/priming tips prior to transfer	+	+		+
	Optimizing sample analysis procedure to limit exposure to adsorption, such as no evaporation (drying down)		+		
	Use of good IS, for example, SIL IS, and at appropriate concentration		+	+	
	Sonication	+	+		+
	Heating or increase temperature	+	+	+	
	Avoiding gradient elution, or optimizing elution gradient, or adding a dummy gradient or sawtooth column wash cycles			+	
	Using tubes/vials of appropriate sizes	+	+	+	+
	Optimizing washing solutions and/or cycles; use of full-loop injection		+	+	
	Injection in concentration-ascending order; injecting a blank or blanks after high concentration samples			+	
Instrumentation or materials	Use of better LC-MS instrumentation, for example, Shimadzu autosampler		+	+	
	Use of better LC columns or cartridges, for example, endcapped, nonporous, and monolith		+	+	
	Good instrument/equipment preventive maintenance		+	+	
	Use of special vials/tubes/plates/tubing or pretreatment first, for example, silanized glass, low binding, and PEEK	+	+	+	+
Others	Conversion, decomposition, or derivatization	+	+	+	+
	Sacrificing/discardng a portion of sample/solution	+	+	+	+

Notes: A “+” sign indicates the applicability of a measure to the stage of bioanalysis. BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HAS, human serum albumin; IS, internal standard; PEEK, polyetheretherketone; SDBS, sodium dodecylbenzenesulfonate; SIL, stable isotope labeled.

like adjusting pH or the ionic strength (Oe et al., 2006; Larsen and Larsen, 2013; Maes et al., 2014b). Still others use sacrificial competing agents that have stronger interactions with the surfaces and/or are present at much higher concentrations, so that the chance of the compound of interest being adsorbed is significantly reduced (Oka et al., 1985; Walsh et al., 1992; Santos et al., 1996; Chen and Bartlett, 2012).

Moreover, efforts can be made to optimize the bioanalytical procedure to eliminate or reduce the impact of NSB. These include priming pipette tips prior to each transfer (Maes et al., 2014b), limiting the number of transfers by using evaporation-free extraction (Tan et al., 2007; 2009), preloading collection plates with an organic solvent prior to the transfer of incubation samples (Cai et al., 2012), maintaining/using an adequate (high) sample volume and concentration, diluting human urine samples in human plasma (Dubbelman et al., 2012), avoiding gradient elution, or adding a “dummy” gradient or sawtooth column wash cycles (Weng and Halls, 2002; Williams et al., 2012; Maes et al., 2015). Finally, other physical measures like heating and sonication (Xu et al., 2012) and change of LC columns or LC-MS instrument platforms may also be adopted (Vu et al., 2013).

It should be mentioned that some measures may reverse NSB losses, that is, recover the “lost” or adsorbed analyte, while others may only prevent NSB losses. In the latter case, the selected measure must be taken prior to the occurrence of NSB. For example, during the method development phase for quantifying BAF312 in human urine, it was found that Tween-80 was able to recover the adsorbed analyte, while  $\beta$ -cyclodextrin was not. Hence,  $\beta$ -cyclodextrin must be mixed to control blank urine before the analyte is spiked in the human urine matrix, whereas Tween-80 may be added either prior to or post-CS/QC preparation (as long as the container is not changed) (Li et al., 2010).

#### 9.4.3 Application Examples

Despite a wide variety of measures at our disposal to tackle NSB issues, finding the right solution for the NSB issue at hand may sometimes be quite challenging. It requires a full understanding of the problem at hand, for example, the physicochemical properties of the compound of interest, the matrix, the instrumental principle, and the various materials involved, as well as thoughtful and rational design of the bioanalytical procedure, followed by experimental evaluation of the effectiveness of the adopted measure(s). The final adopted measure(s) should ideally be easy to implement and cost effective and cause minimal interference with the quantification, such as ion suppression or enhancement by the added surfactant (Chen et al., 2009; Ji et al., 2010).

The following are several real case examples from the LC-MS bioanalysis of both small and large molecules.

The first one is a very challenging NSB issue that we experienced during the method development phase for a highly hydrophobic basic small molecule compound in rat EDTA plasma. Chromatographic peak tailing, excessive carry-over, variable recovery, upward nonlinear calibration curve, and poor precision and accuracy were all observed. Through extensive troubleshooting, a suite of measures were finally found and all the issues were successfully resolved. The chromatographic peak tailing was minimized by using a basic mobile phase, in which the charge of the analyte was neutralized so that the ionic interaction between the residual silanol group and the analyte did not occur. The excessive carry-over was reduced by using isocratic elution together with high organic acidic and basic washings, methanol/H<sub>2</sub>O (80/20, v/v) + 10 mM ammonium acetate + 0.5% (v/v) formic acid and methanol/H<sub>2</sub>O (80/20, v/v) + 10 mM ammonium acetate + 0.5% (v/v) ammonium hydroxide. The nonlinearity was determined to be caused mainly by NSB losses during the preparation of working standard solutions and to a less extent by NSB losses in the prepared matrix samples. Since the salt form reference standard dissolves faster in methanol than in acetonitrile, the stock solutions were prepared in methanol. However, increased adsorption losses at low concentration working standard solutions (methanol based) were noticed. Changing the solvent for working standard solutions from methanol to acetonitrile and maintaining a minimum plasma sample volume of 200  $\mu$ L per tube straightened the calibration curve. The variable recovery and poor precision issue was resolved by maintaining a sufficiently high percent of organic in the reconstitution solution and the working IS solution. In the end, a very accurate and precise bioanalytical method was successfully validated and applied to a toxicity study with a 100% incurred sample reanalysis (ISR) passing rate.

The second example is for the quantification of teriparatide (human parathyroid 1–34) in human plasma by LC-MS/MS (Chambers et al., 2013). In order to achieve a clinically relevant LLOQ between 10 and 50 pg/mL, the authors had to overcome several important challenges, including poor solubility, severe NSB losses, and low recovery. Teriparatide has 34 amino acid residues (SVSEIQLMHN LGKHLNSMER VEWLRKKLQD VHNF) and it is a basic polypeptide (overall net charge: +1) with 38 and 41% of hydrophobic and charge residues, respectively. Accordingly, the authors prepared the stock solution in DMSO and working standard solutions in H<sub>2</sub>O/acetonitrile/formic acid (80/19/1, v/v/v) containing 0.05% rat plasma (in our opinion, as per Table 9.2, the stock may also be prepared in the same solvent as the working standard solutions). The addition

of rat plasma in the working standard solutions together with the use of LoBind tubes reduced the NSB losses during standard solution preparations. The sample extraction was based on protein precipitation (PPT) followed by SPE. Several different measures were combined to reduce the NSB losses, improve the solubility, and increase the recovery during the sample extraction. For example, to avoid the potential precipitation of the analyte (a large peptide) and to disrupt protein binding more efficiently, plasma samples were precipitated with acetonitrile containing 5% (v/v) NH<sub>4</sub>OH in a solvent to plasma ratio of 1:1, instead of the normally used 3:1 ratio. In addition, trifluoroethanol (TFE) was added in the elution solvent to improve the solubility, and an evaporation-free approach (Tan et al., 2007, 2009) was adopted to reduce NSB losses and avoid the resolubilization issue. For LC separation, the use of a charged surface hybrid (CSH) column together with the use of weak acidic mobile phases greatly reduced the chromatographic peak tailing. The low level positively charged moiety on the C18 column particle surface made it difficult for the positively charged analyte to be diffused into small pores, which avoided extra peak broadening and tailing. All of these measures together resulted in an accurate and precise method with an LLOQ of 15 pg/mL for a relatively large peptide.

Additional representative examples are presented in Table 9.4. As the examples show, there is no universal one-size-fits-all solution to deal with NSB issues. Each case has its own unique challenges. Accordingly, the solution to each case is usually different. Nevertheless, the collective success of these various exemplary cases demonstrate that no matter how challenging an NSB issue may be, a unique solution can always be found. The key to success is a good understanding of the problem together with rational experiment design.

## 9.5 Conclusion

NSB is an undesirable yet very common phenomenon in LC-MS bioanalysis. An upward turning calibration curve, low and variable recovery, “instability” or poor accuracy at low concentration end, chromatographic peak tailing, and carry-over could all indicate a potential NSB issue. In addition, low protein matrices (e.g., urine and CSF) and a wide variety of analytes, like peptides, oligonucleotides, tetracyclines, basic, hydrophobic, and strongly protein-binding compounds, are prone to NSB issues. Therefore, whenever the aforementioned matrices or analytes are involved, efforts should be made to check for NSB.

To confirm NSB or evaluate the severity, many different approaches can be taken, such as multiple sequential transfers, the deliberate preparation of small and large volumes, and frequent comparisons with fresh spiking. The key behind these various approaches is to amplify the impact of NSB by exposing the compounds of interest to as large of a surface area as possible and by exposing as many times as possible, so that it will not go unnoticed. As the impact of NSB depends on many factors like sample matrix, volume, concentration, surface area and surface activity of the container, and temperature, any evaluation should be conducted at conditions specific to the task at hand. Moreover, NSB should not be confused with a stability issue, though both can lead to a similar negative bias for quantification. NSB and stability issues can be distinguished in terms of their dependence on sample volumes, concentrations, time, and materials, as well as the reversibility of losses.

The fundamental causes for NSB are often inadequate solubility and/or relatively stronger interactions (hydrophobic, ionic, van der Waals, hydrogen bonding, etc.) of the compounds of interest with various surfaces than those with bulk solvents or matrices. NSB could be especially problematic for the LC-MS bioanalysis of biomarkers because some of the common matrices (urine, CSF, etc.) and analytes (peptides and proteins) involved in biomarker analysis are especially prone to NSB issues, and often more than one cause coexists, which dictate the simultaneous use of multiple measures.

A wide variety of measures are available to overcome NSB challenges, from using additives and optimizing bioanalysis procedures to selecting better instrumentation and materials. The proper solubilization of the compounds of interest at all stages of sample analysis is the prerequisite for eliminating or reducing NSB. However, the competitive nature of NSB between surfaces and bulk solvent/matrix must not be overlooked. Depending on the physicochemical properties of the compounds of interest and the symptoms, it is critical to use rational thinking/reasoning to design the most effective solution for the NSB issue at hand. As long as this approach is taken, a unique solution can always be found for each and every challenging case.

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**Table 9.4** Representative examples for eliminating NSB or reducing its impact in LC–MS bioanalysis.

Analyte	Matrix	Symptom	Key measure	Reference
Cyclic hexapeptide (L-743 872)	Human urine	Loss due to adsorption to plastic surfaces	Addition of BSA to a final concentration of 0.5%	Schwartz et al. (1997)
BMS-708163 ( $C_{20}H_{17}ClF_4N_4O_4S$ )	Human CSF	Upward calibration curve; adsorption to collection tubing	No evaporation; pre- or post-spiking of 0.2% Tween-20; discard the 1st 2 mL during sample collection	Gu et al. (2010)
Saxagliptin and 5-monohydroxy metabolite	Human plasma	High negative bias values for LLOQ and low QC s	Addition of CHAPS + sonication + heat	Xu et al. (2012)
Triclosan	Human urine	Adsorption loss for free form, not for glucuronide	Use of glass tubes and addition of acetonitrile	Provencher et al. (2014)
Amyloid $\beta$ peptides	Human CSF	Readily self-aggregate or bind to other proteins and glassware	$^{15}N$ -labeled amyloid $\beta$ peptides as ISs; basic MP and basic solvents for dissolution of the peptides and for washing LC injection needle	Oe et al. (2006)
Amyloid $\beta$ peptides	Dog CSF	Adsorption to solid surface and carry-over	Dilution of dog CSF in $H_2O$ /acetic acid/methanol (2:6:1, v/v/v) and direct loading on peptide adsorption-controlled (PAC) LC–MS, a specially designed solvent gradient that allow reversible conformational change of peptides	Goda and Kobayashi (2012)
Neuropeptides	Micro-dialysate	Adsorptive losses	Systematic evaluation and optimization of sample/solvent compositions, tubes, pipette tips, vials, and pipetting protocols for each stage of analysis to reduce losses and increase sensitivity	Maes et al. (2014b)
Pasireotide (a cyclic peptide)	Monkey plasma	Adsorption loss, chromatographic peak tailing and LC column carry-over	Direct injection of diluted SPE eluent; addition of TFA and acetic acid in MP	Fu et al. (2016)
Antibody-conjugated payload	Monkey plasma	Adsorption of ADC to collection plate; carry-over of payload during LC	Addition of 0.5% BSA to elution buffer to minimize adsorption; starting LC gradient with high organic together with both basic and acidic washes for autosampler	Liu et al. (2015)
Protein therapeutic (BMS-A)	Monkey plasma	Adsorption losses	Addition of 1% HSA in stock solutions; preparation of post-digestion spiking solutions in digested blank	Zhao et al. (2015)
Tetracyclines	Animal tissues	Low recovery from homogenization and SPE; chromatographic peak tailing; carry-over	Addition of EDTA or oxalic acid in homogenization solvent, elution solvent, and MP	Oka et al. (1985), Santos et al. (1996), Walsh et al. (1992)
Oligonucleotides	Rat or human plasmas	Adsorption to container, SPE cartridges, LC column and system; low recovery; carry-over	Addition of EDTA or BSA in MP, neat washing, or sample extraction solutions; use of silanized vials; adequately high IS concentration	Chen and Bartlett (2012), Wang (2011), Zhang et al. (2007)
Ritonavir	Human plasma	Carry-over	Use of isocratic elution, instead of gradient elution	Weng and Halls (2002)
A weak acidic compound with F, Cl, and OH on the same benzene ring	Human plasma	Carry-over	Use of full-loop injection, instead of partial-loop injection, for a more effective flushing of sample flow path	Vallano et al. (2005)
Various	n/a	Elution gradient and/or column related carry-over	Use of columns that are less prone to carry-over, for example, nonporous or polymer monolith stationary phases; cycling washings of column with high and low organic MP s, instead of continuous wash; dynamic back flushing of column	Garcia-Cañas et al. (2006), Dolman et al. (2013), Vu et al. (2013), Williams et al. (2012), Maes et al. (2015), Chen et al. (2014)
S-Adenosyl-L-methionine	Plasma	Carry-over	Derivatization of residual analyte with propionic anhydride in HPLC system	Vasquez et al. (2015)
Various	CSF	Loss to sample collection tubing	Recommended to use Pharmed <sup>®</sup> collection tubing, instead of silicone tubing	Wagdy et al. (2011)

Notes: ACN, acetonitrile; ADC, antibody drug conjugate; BSA, bovine serum albumin; CSF, cerebrospinal fluid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FA, formic acid; HSA, human serum albumin; IS, internal standard; LLOQ, lower limit of quantitation; MP, mobile phase; SPE, solid-phase extraction; TFA, trifluoroacetic acid.

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## 10

### Strategies for Improving Sensitivity for Targeted Quantitation by LC–MS

Long Yuan and Qin C. Ji

*Bioanalytical Sciences, Bristol-Myers Squibb Co., Princeton, NJ, USA*

#### 10.1 Introduction

Sensitivity is a critical parameter for any analytical method. For an LC–MS bioanalytical method, the sensitivity is often referred to as the lower limit of quantification (LLOQ), the lowest concentration of the analyte that can be quantified in biological samples with acceptable accuracy and precision. Strictly speaking, sensitivity and quantification limit are two different concepts. Sensitivity represents the ability of a method to distinguish between small differences in concentration of a given analyte in a sample at a desired confidence level (Fifield, 2000). A simple measurement of sensitivity is the calibration sensitivity, the slope of the calibration curve in the concentration range of interest. Steeper calibration curve means better sensitivity for a given sample standard deviation. For an analytical method having a linear calibration curve, its sensitivity is constant over the whole curve range. While for a method having a nonlinear curve (e.g., quadratic regression), the sensitivity changes over the range and may have decreased sensitivity at the high concentration end of the curve. For the convenience of description, in the remainder of this chapter, sensitivity is interchangeably used as quantification limit or LLOQ.

Because the term “sensitivity” is used in various ways in the literature, it is important to define how sensitivity or quantification limit is determined. Based on the US Food and Drug Administration (FDA) Guidance for Industry: Bioanalytical Method Validation (Food and Drug Administration, 2001) and the European Medicines Agency (EMA) Guideline on bioanalytical method validation (European Medicines Agency, 2011), for LC–MS-based bioanalytical methods measuring drug concentrations, the analyte signal of the LLOQ sample should be at least five times greater than the signal of a blank sample. In addition, the accuracy and precision of the analyte response should be within the desirable

range (e.g., a precision of 20% and an accuracy of 80–120% for a validated method at the LLOQ level). The lowest calibration standard in the calibration curve is usually set at a concentration that corresponds to the LLOQ of the method. These requirements were developed for methods measuring drug concentrations and not for those measuring biomarker concentrations. However, the same principles can still be applied to biomarker assays, although the required signal/noise (S/N) ratio, accuracy, and precision may be different based on the needs of individual assays, and fit-for-purpose assay establishment strategy should be used (Lee et al., 2006).

The sensitivity of LC–MS bioanalytical methods has increased extraordinarily over the past three decades. In the 1990s, reaching an LLOQ at the low ng/mL and pg/mL level for a small molecule analyte was considered very challenging. Nowadays, with advances in sample preparation techniques, chromatography columns, and LC–MS instrumentation, the low ng/mL or even pg/ml level of LLOQ can be readily achieved. Reaching an LLOQ in the low pg/mL level is still a more challenging task. Innovation in drug discovery and development (e.g., application of new ideas, approaches, and technologies) has been a major driving force for increasing the sensitivity of analytical methods. For example, in recent years, LC–MS assays have gained greatly increased interest for the quantification of proteins due to characteristics of LC–MS assays such as high selectivity, reduced interference, wide linear dynamic range, and the ability to quantify multiple proteins simultaneously (Ezan and Bitsch, 2009; Li et al., 2011a; van den Broek et al., 2013; Yuan et al., 2015). However, one major limitation of LC–MS assays for large biomolecules is their often poor sensitivity compared to ligand binding assays. Improving sensitivity has therefore become a critical factor for the wider application of LC–MS in protein bioanalysis. Especially, protein biomarkers are often present at low levels, demanding superior sensitivity for quantitative

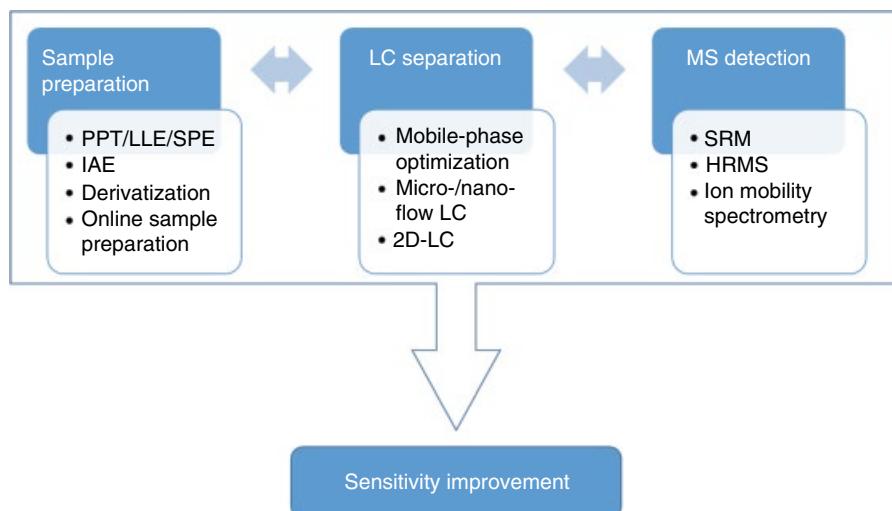
biomarker assays. Other good examples where high assay sensitivity is required include the application of microdosing in absolute bioavailability studies and the use of microsampling techniques (e.g., sampling in capillary tubes or dried blood spot (DBS)) for blood/plasma sample collection. These demands have greatly boosted the need and development of new methodologies or strategies for improving sensitivity. On the other hand, the progress of the new methodologies or strategies will also further enable and drive the wider application of these novel techniques/approaches.

There are three major components for an LC-MS bioanalytical assay: sample preparation, LC separation, and mass spectrometry (MS) detection. All these components can be optimized to improve the sensitivity of an assay. As shown in Figure 10.1, various strategies or technologies can be applied in these processes to increase the signal of the analyte and/or reduce the background noise. Sometimes, different strategies from these components may not all work well for the sensitivity improvement. Depending on the assay requirements and the availability of instrumentations, trade-off can be made among these three areas to get an appropriate strategy. For example, if a highly selective sample preparation method is used, a short chromatographic separation may be good enough to achieve the desired sensitivity. If limited sample purification can be performed, more extensive optimization of the LC separation and MS detection components would be needed. To achieve supersensitive assays, these different strategies need to be systematically evaluated and effectively incorporated into a single method. In this chapter, strategies and technologies for improving sensitivity for various types of analytes,

including small molecules, peptides, and proteins, will be introduced. The strategies discussed here can be applied to not only the LC-MS analysis of biomarkers but also the analysis of drugs, metabolites, and other analytes.

## 10.2 Sample Preparation Strategies for Improving Sensitivity

Sample preparation, the first step for an LC-MS method, is critical for improving the sensitivity. One commonly used practice to improve sensitivity is to increase the volume of sample used for extraction, or the injection volume. This simple and easy approach works very well for a neat standard solution. However, for the real biological samples, it may not achieve the desired sensitivity improvement if lacking an appropriate sample preparation process to obtain clean sample extracts (Aubry, 2011). Sometimes, it could even cause the decrease of the sensitivity, since increasing the sample volume or injection volume also increases matrix components in the extracted sample (Yuan et al., 2013). In addition to the target analyte, various endogenous components (e.g., proteins, lipids, salts) are present in biological samples, and they usually are much more abundant than the analyte. If these constituents are carried through in the extracted samples and co-elute with the analyte, they can cause suppression, or enhancement in some cases, of the analyte ionization (matrix effect) during MS analysis, especially when using electrospray ionization (Matuszewski et al., 2003). Matrix effect can severely impact the sensitivity, accuracy, and precision



**Figure 10.1** Major components of an LC-MS bioanalytical method, and the various strategies for improving sensitivity corresponding to the three components. HRMS, High-resolution mass spectrometry; IAE, immunoaffinity extraction; LLE, liquid–liquid extraction; PPT, protein precipitation; SPE, solid-phase extraction; SRM, selected reaction monitoring.

**Table 10.1** Comparison of different sample preparation strategies.

	Removal efficiency			Overall purification efficiency	Pros	Cons
	Proteins	Lipids	Salts			
PPT	+++	+	+	+	Easy and fast operation; low cost	Limited sample purification
LLE	+++	++	+++	++	Relatively clean extract; low cost	Not good for polar compounds, peptides, and proteins
SPE	+++	++	++	++	Various separation mechanisms to select	Requires extensive method development; relatively high cost
IAE	+++	+++	+++	+++	Highly specific purification	Requires specific reagent; high cost

IAE, immunoaffinity extraction; LLE, liquid–liquid extraction; PPT, protein precipitation; SPE, solid-phase extraction.

+, low; ++, moderate; +++, high.

of LC-MS bioanalytical assays. Recovery is another important factor that needs to be considered for a proper sample preparation strategy, since, generally, the higher the recovery, the better would be the sensitivity. An ideal sample preparation method should be able to recover 100% of the analyte from the biological sample and remove all the matrix components, therefore maximally improving the sensitivity. However, it is difficult to achieve ideal sample preparation in real applications. A sample preparation method that can extract most of the analyte may also extract abundant matrix components and result in severe matrix effect (if not resolved by chromatography). Or vice versa, a method could generate very clean sample extracts, but have low recovery of the analyte. Therefore, to improve sensitivity, both matrix effect and recovery should be evaluated to achieve an optimal sample preparation method, which is often a compromise of them.

For small molecule analytes, proteins and salts are relatively easy to remove from biological samples. Phospholipids, due to their amphiphilic nature with both hydrophobic (the two fatty acid “tails”) and hydrophilic (the phosphate “head”) functional groups, are usually difficult to eliminate. The abundant phospholipids in plasma have been shown to be one major source of the matrix effect for small molecule bioanalytical LC-MS assays (Jemal et al., 2010). As a result, ensuring the effective removal of phospholipids during sample extraction has become a common practice for improving the sensitivity, as well as the quality and ruggedness, for small molecule bioanalytical assays. For LC-MS bioanalysis of protein analytes, the high abundance endogenous proteins (e.g., serum albumin, immunoglobulins) in biological samples are the major source of matrix effect. The concentrations of these endogenous proteins in plasma/serum are usually much higher compared with that of the protein analyte. Some of these proteins (or their resulting peptides after digestion) may

have similar physicochemical properties to the protein analyte (or its corresponding surrogate peptide), making it difficult to separate them from the protein (or peptide) of interest, and may cause severe ion suppression, high background noise, and potential interference with the LC-MS analysis, therefore severely reducing the assay sensitivity (Ewles and Goodwin, 2011; Li et al., 2011a; Yuan et al., 2015).

Various sample extraction strategies have been developed. This chapter will focus on the ordinarily used sample preparation strategies in drug research and development, as these are more applicable and practical to be used in real studies. For small molecule analytes, protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE) are the most commonly used sample preparation methods. For peptide and protein analytes, SPE, PPT, and immunoaffinity extraction (IAE) are widely used (John et al., 2003; van den Broek et al., 2008; Ewles and Goodwin, 2011; Li et al., 2011a; van den Broek et al., 2013; Yuan et al., 2015). The advantages and limitations of these sample preparation strategies are summarized in Table 10.1 and will be described in more detail next.

### 10.2.1 Protein Precipitation

In PPT, a denaturing agent is added to the biological sample, causing most of the proteins to precipitate out of solution. The solid and liquid components are then separated, for example, by centrifugation, and the liquid supernatant is analyzed (Chang et al., 2007). PPT is simple, fast, and cost effective and usually can achieve a very good recovery, particularly for small molecule analytes. However, due to its limited ability in sample cleanup, significant amounts of matrix components will still be present in the resulting supernatant, which could cause severe matrix effect to the analyte and affect the sensitivity of the assay. Furthermore, there is no element of sample

concentration during PPT (indeed, the sample is often diluted!). Therefore, PPT usually is not the first choice for improving assay sensitivity. PPT may be advantageously used with strongly protein-bound analytes since it can effectively dissociate the binding and release the analyte from the proteins (Yang et al., 2004), therefore increasing recovery and maximizing sensitivity. In addition, since PPT is much faster than LLE or SPE and can use a mild neutral extraction condition (LLE or SPE may require an acidic or basic condition), it is often used for the extraction of unstable compounds (Yuan et al., 2014a). PPT can also be easily combined with other sample preparation methods. For example, vitamin D and its metabolites mainly present in protein-bound forms in plasma or serum. For their analysis, often a PPT step is first applied to release the analytes from vitamin D binding proteins; then, this is followed by an additional LLE step to further purify the samples (Zhang et al., 2014a; Wang et al., 2015).

PPT can also be used for the purification of peptides and organic soluble proteins. For example, PEGylated proteins (Wu et al., 2011; Dawes et al., 2013) and small proteins (Becher et al., 2006; Zhang et al., 2014b) have a certain degree of solubility in the water-miscible organic solvents used for PPT (e.g., acetonitrile, methanol, and isopropanol). With appropriate optimization of the PPT conditions, these molecules can be efficiently retained in the supernatant and easily separated from the precipitated background proteins. Zhang et al. (2014b) achieved high recovery (almost 100%) and minimum matrix effect for the extraction of interferon-gamma-inducible protein-10 (molecular weight (MW), 8.6 kDa) from human serum using PPT with optimized acidic precipitation solvent and solvent-to-serum ratio. As a result, they obtained a highly sensitive LC-MS/MS method with an LLOQ of 31.62 pM, which was the same as analyzing the neat sample in buffer and 100-fold better than using a direct digestion method. PPT is not efficient for the extraction of large proteins (e.g., a monoclonal antibody (mAb)), since large proteins mostly would coprecipitate with endogenous proteins in serum/plasma samples (Yuan et al., 2012). Recently, Liu et al. (2014) developed a novel acid-assisted PPT method that can efficiently remove albumin from plasma/serum samples. Albumin was retained in the supernatant and was removed, while large target proteins in the samples were precipitated and then resuspended for further processing. They evaluated the combinations of four organic solvents (acetone, methanol, ethanol, and isopropanol) and two acids (formic acid and trichloroacetic acid) at different concentrations as the precipitation solvent. They found that a combination of isopropanol with 1.0% trichloroacetic acid could remove 95% of total albumin from plasma and achieved satisfactory recoveries (>60%) for the three

therapeutic proteins tested (a mAb, a domain antibody, and a fusion protein). This new method provided much cleaner extracts and thus a significantly improved assay sensitivity (10-fold for the therapeutic protein tested) compared with a conventional PPT method. They have successfully validated an LC-MS/MS method for a mAb using this new approach and applied it to a monkey toxicology study (Zhao et al., 2015).

### 10.2.2 Liquid-Liquid Extraction

In LLE, a two-phase system (usually an aqueous solution and an immiscible organic solvent or solvent mixture) is set up to utilize the differential distribution of an analyte, typically at its non-charged status by adjusting the pH prior to the extraction, between phases. By appropriate choice of the acceptor phase composition and volume, the desired analyte can be selectively extracted and possibly concentrated (Chang et al., 2007). LLE can efficiently remove proteins, salts, and lipids from biological samples and usually generates much cleaner extracts than PPT. It is also easy to be automated in a 96-well plate format, which enables simple operation, high-throughput extraction, and this is achieved at a relatively reduced cost compared with SPE. Therefore, LLE has been a common and often preferred sample preparation method for achieving a clean sample extract. To achieve the best combination of minimal matrix effect and maximum recovery for the analyte, the LLE conditions (e.g., organic extraction solvents, extraction buffer, pH, and ratio of sample and extraction solvent volumes) need to be carefully evaluated. Sometimes even the duration of extraction time could significantly affect the recovery and matrix effect of the method (Aubry, 2011). Various water-immiscible organic solvents (e.g., ethyl acetate, methyl tertiary-butyl ether, *n*-butyl chloride, hexane, and toluene) can be used for LLE. It would be inefficient and time consuming to screen all the different solvents with different combination of extraction buffer and pH. A simple approach is to screen mixtures of ethyl acetate and hexane with different volume percentages as the extraction solvent, and extraction buffers at different pH (acidic, neutral, and basic) (Yuan et al., 2016). This approach can cover analytes with a wide range of polarity: with hexane offering good extraction efficiency for nonpolar compounds and ethyl acetate for moderately polar compounds. One limitation of LLE is its poor applicability for polar compounds: these compounds are often more soluble in water and cannot be extracted into the organic phase with good recoveries. For the same reason, LLE is rarely used for the extraction of peptides and proteins.

Salting-out assisted liquid–liquid extraction (SALLE) utilizes the salting-out effect of concentrated salt

solution (e.g., magnesium sulfate, ammonium acetate) in water-miscible organic solvent (e.g., acetonitrile, tetrahydrofuran); thus, the organic solvent containing the target analyte can be “salted out” and separated from the aqueous phase (Tang and Weng, 2013). Compared with the conventional LLE methods, SALLE is more efficient in extracting polar compounds. SALLE also provides cleaner extracts than PPT, since high concentration salt along with the organic solvent can effectively precipitate proteins in biological matrices. SALLE has been applied for the extraction of a variety of compounds. For polar compounds that are difficult to be extracted using traditional LLE or SPE methods, SALLE could be a useful approach for improving sensitivity.

### 10.2.3 Solid-Phase Extraction

SPE is a chromatographic separation-based extraction technique that offers a variety of separation mechanisms (e.g., reversed phase (RP), ion pair, ion exchange, mixed mode) through the use of different sorbents. As a result, SPE can provide efficient extraction for various types of analytes, including both nonpolar and polar compounds, peptides, and intact proteins. To obtain a highly sensitive assay, it is better to choose a separation sorbent and mechanism that is selective for the analyte. The more selective the SPE method, the cleaner the resulting sample extract, and the better the sensitivity that can be obtained. This approach is particularly useful for analytes with unique physicochemical properties that can be exploited by choice of an appropriate SPE phase. For example, for ionizable compounds, a sorbent that uses an ion exchange separation mechanism can be highly selective and, therefore, can result in highly efficient sample purification and significantly improved sensitivity. This has been demonstrated in the extraction of basic compounds by using strong cation exchange (SCX) SPE (Shen et al., 2005; Xue et al., 2006). In addition, SPE can easily preconcentrate or enrich the samples, which is often inconvenient for an LLE or PPT method in a 96-well plate format. For instance, a large volume of sample (e.g., 1 mL or even more) can be loaded to the SPE plate or cartridge, and after washing, the analyte can be eluted and reconstituted in a much smaller volume (e.g., 100 µL) of solvent, which would result in significant (10-fold or more) enrichment of the samples. This preconcentration is a very useful approach for improving sensitivity, especially when enough volume of sample is available.

SPE has also been widely used for the purification of peptides and proteins from biological matrices. The selection of a suitable SPE sorbent mainly depends on the polarity and charge (isoelectric point, pI) of the analyte. RP sorbents are the most commonly used, while the utilization of ion exchange materials (e.g., SCX (Zhang

et al., 2007; Yuan et al., 2013)) and mixed-mode anion exchange (Halquist et al., 2012; Gong et al., 2015) has also been reported. For small proteins (Ji et al., 2003, 2007; Ruan et al., 2011) and PEGylated proteins (Yang et al., 2009; Li et al., 2011c), SPE sample preparation can efficiently remove most of the endogenous background proteins (such as albumin and immunoglobulins) and retain the intact protein analyte in the extracts. For large proteins such as monoclonal antibodies (mAbs), it is difficult to directly separate them from endogenous proteins by SPE. Alternatively, SPE can be applied for the extraction of the surrogate peptides of the digested target protein (Yang et al., 2007; Heudi et al., 2008; Yuan et al., 2013; Bronsema et al., 2015). An effective way to achieve better sample purification is to use an SPE separation mechanism orthogonal to the chromatographic separation (Yuan et al., 2013). An orthogonal sample preparation with a different mechanism of separation to the chromatographic separation can usually achieve better sample purification than using SPE with the same separation mechanism as in the analytical separation. For an LC-MS method with RP chromatographic separation, SCX SPE, RP SPE, and two-dimensional (2D) SPE (RP SPE followed by SCX SPE) were systematically compared for the extraction of digested serum samples (Yuan et al., 2013). SCX SPE, which was orthogonal to the RP chromatographic separation, was more efficient than RP SPE in removing the background peptides in serum tryptic digests. Although 2D SPE provided additional cleanup of the sample, the improvement was not significant compared with using SCX SPE alone.

Amino acids such as histidine, cysteine, and tryptophan have strong electron donor groups that can chelate with transition metals such as Ni<sup>2+</sup>, Zn<sup>2+</sup>, and Co<sup>2+</sup>. By utilizing this chelating effect, immobilized metal affinity-based extraction methods can selectively extract peptides or proteins with these types of amino acids exposed on the surface (Kokubu et al., 2005; Zheng et al., 2005). Wilffert et al. (2013) applied immobilized metal affinity chromatography (IMAC) for the extraction of recombinant human TNF-related apoptosis-inducing ligand (rhTRAIL), a 60 kDa protein that contains a number of surface exposed histidines, in the serum. The strong interaction between histidines of rhTRAIL and the immobilized Ni<sup>2+</sup> ions on the IMAC resin enabled effective purification of the samples: 95% of serum proteins were removed, and rhTRAIL was well retained with an extraction recovery of 72%.

2D SPE, with an additional dimension of cleanup, can be applied to achieve further purification of the peptides of interest. Wilffert et al. (2016) added an SCX SPE step before the IMAC SPE step for the quantification of rhTRAIL in the serum. This 2D SPE approach removed >99.9% of serum protein. Although the overall recovery of rhTRAIL decreased from 72 to 49%, the much cleaner

extracts enabled a >10-fold increase in sensitivity. In another example, Yang et al. (2007) applied RP SPE followed by SCX SPE for the analysis of a therapeutic mAb in the serum. The RP SPE efficiently eliminated salts and highly hydrophobic constituents, and the SCX SPE removed background peptides with basicity largely different from the peptides of interest and therefore significantly reduced the background noise, ion suppression, and interference and improved the sensitivity in LC-MS/MS analysis.

#### 10.2.4 Immunoaffinity Extraction

IAE utilizes an immunoaffinity capturing reagent (e.g., antibody or the receptor/ligand of the analyte) to selectively and efficiently capture the analyte (protein, peptide, or small molecule compound) of interest from biological samples. For protein bioanalysis, due to the presence of high abundance endogenous proteins (or background peptides after digestion) in the samples, conventional sample preparation method such as SPE often cannot provide sufficient sample cleanup to achieve the desired sensitivity. IAE, with its excellent selectivity, is highly suitable for the extraction and enrichment of proteins or peptides from complicated biological matrices. In recent years, immunoaffinity-based sample preparation methods have drawn tremendous interest and attention for quantitative LC-MS bioanalysis of proteins. When an appropriate capturing reagent is available, IAE is undoubtedly the most effective sample preparation method to achieve an ultrasensitive method, often reaching LLOQs at low ng/mL levels for protein analytes. The purification efficiency of IAE depends on the selectivity of the capturing reagent used in the assay: the more selective the reagent, the better the enrichment and purification that can be achieved for the target analyte, and as a result, the higher the assay sensitivity.

Protein A or protein G can selectively bind to the fragment crystallizable region (Fc region) of immunoglobulin G (IgG) and, hence, can be used for the IAE of antibodies containing the IgG Fc region. However, they can only provide limited sample purification and sensitivity improvement (Ocaña et al., 2012), since they cannot differentiate the endogenous IgGs in plasma/serum from the analyte of interest. An antihuman IgG antibody can provide much improved selectivity compared with protein A or protein G, as it only binds to the Fc region of human IgGs, and not IgGs from other species. It can purify various human mAbs from animal matrices with high efficiency and selectivity and therefore significantly improve the sensitivity. This has become a generic immunocapture method for the extraction of human mAbs in animal plasma/serum (Li et al., 2012) and tissue (Sleczka et al., 2014) and can also be used to simultaneously extract multiple mAbs (Liang et al., 2013).

To obtain even cleaner sample extracts, capturing reagents with better selectivity are required for IAE. There are two commonly used strategies for the highly specific immunocapture of proteins and peptides. One strategy uses anti-idiotypic antibodies or the receptor/ligand of the target analyte to achieve highly selective purification and effective enrichment of the intact analyte. This approach has been widely applied for various protein analytes, including protein or peptide biomarkers (Berna et al., 2006; Oe et al. 2006; Winther et al., 2009; Ocaña and Neubert, 2010; Rafalko et al., 2010; Torsetnes et al., 2014). Another frequently used approach is the anti-peptide approach named “stable isotope standards and capture by anti-peptide antibodies” (SISCAPA), which was first developed by Anderson et al. (2004). In this strategy, the sample containing the protein (or large peptide) analyte is first digested to peptides, and then an anti-peptide antibody is used to specifically capture the signature peptide of the analyte in the digested sample. This approach has been applied in various matrices, including plasma/serum (Anderson et al., 2004; Whiteaker et al., 2007; Wang et al., 2012), saliva (Neubert et al., 2010), cell lysates (Schoenherr et al., 2012; Whiteaker et al., 2015), tissue (Schoenherr et al., 2012; Whiteaker et al., 2015; Fan and Neubert, 2016), and DBS (Razavi et al., 2016). The anti-peptide immunocapture is highly selective and efficient. Usually one can obtain extremely clean sample extracts with >100-fold enrichment; therefore, this could result in sensitivity enhancements of about two orders of magnitude (Anderson et al., 2004; Whiteaker et al., 2007). Compared with the previously described anti-intact analyte approach, this strategy eliminates any potential interference from antidrug antibodies (ADAs), soluble target, or other anti-target protein antibodies, since the samples are digested before the extraction.

To further improve the sensitivity, sequential immunocapture of the target protein and its signature peptide can be applied to generate ultraclean and highly enriched sample extracts (Neubert et al., 2012; Palandra et al., 2013). For example, Neubert et al. (2012) first used a magnetic bead-based anti-nerve growth factor (NGF) polyclonal antibody to extract NGF from serum samples. The sample extracts were digested by trypsin and then further purified online using a polyclonal anti-peptide antibody generated against the tryptic NGF signature peptide. They achieved a highly sensitive assay (LLOQ 7.03 pg/mL) for the quantification of human  $\beta$ -NGF in serum using this sequential immunocapture approach. The method was successfully validated and has been implemented in large-scale sample analysis for multiple clinical studies (Schultz et al., 2016).

IAE has also been used for the purification of small molecule analytes (Cai and Henion, 1996; Ferguson et al., 2001; Radabaugh et al., 2008; Hosogi et al., 2010; Yuan

et al., 2011), although not as commonly as it is used for proteins and peptides. For biomarkers, there may be isobaric endogenous compounds generated from the related biological pathways, which could cause interference to the LC-MS/MS analysis of the analyte and affect the sensitivity of the assay. IAE can efficiently eliminate isobaric interferences from the sample. In the LC-MS/MS analysis of 1 $\alpha$ ,25-dihydroxyvitamin D in the serum, severe interference from the matrix components was observed: several isobaric interference compounds coeluted with the analyte and they had the same selected reaction monitoring (SRM) channel (Yuan et al., 2011). Conventional sample preparation methods were not able to completely remove or separate these interference compounds. By applying an IAE step using a commercially available kit for the extraction of 1 $\alpha$ ,25-dihydroxyvitamin D, all the interference peaks were successfully removed, and an ultrasensitive method with LLOQ of 3.4 pg/mL for 1 $\alpha$ ,25-dihydroxyvitamin D3 and 3.9 pg/mL for 1 $\alpha$ ,25-dihydroxyvitamin D2 was achieved.

#### 10.2.5 Chemical Derivatization

Chemical derivatization is a commonly used strategy for improving the sensitivity of LC-MS methods (Iwasaki et al., 2011; Xu et al., 2011; Niwa, 2012; Zhu et al., 2015). Derivatization has been applied for the sensitive analysis of various types of biomarkers, including lipids (Li and Franke, 2011; Han et al., 2015; Canez et al., 2016), steroids (Marcos and Pozo, 2015; Higashi and Ogawa, 2016), amino acids (Chen et al., 2014; Rebane et al., 2015), nucleotides (Nordström et al., 2004; Flarakos et al., 2005), and saccharides (Trim et al., 2015). There are two major derivatization strategies for sensitivity improvement. One approach is for poorly ionizable compounds. By incorporating a charged (e.g., quaternary ammonium) or easily ionizable moiety (e.g., tertiary amine) into the molecule, the MS response of the analyte can be greatly enhanced. For example, testosterone, a neutral steroid hormone, has very poor ionization efficiency in electrospray ionization (ESI) MS. Star-Weinstock et al. (2012) utilized a novel permanently charged quaternary aminoxy reagent to derivatize the ketone functionality of testosterone, which resulted in a significantly improved MS sensitivity. The detection limit of the derivatized testosterone was improved 80-fold compared with that of the non-derivatized analyte. Another approach is mainly for polar compounds (e.g., amino acids and carboxylic acids) that often have poor retention on the RP column. Introducing a hydrophobic moiety by derivatization can greatly improve their retention on the column so that they elute relatively late under a higher percentage of organic solvent. The derivatized analyte would have significantly improved evaporation, nebulization, and MS

ionization efficiency characteristics and, therefore, a much improved sensitivity. When designing the derivatization reagents, in addition to the hydrophobic moiety (e.g., alkyl chains, ester, or phenyl groups), an ionization enhancement moiety (e.g., tertiary amine) is often included to further increase the MS responses. For instance, various such derivatization reagents (diethyl ethoxymethylenemalonate (DEEMM) (Rebane et al., 2012), isobutyl chloroformate (IBCF) (Li et al., 2013), 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) (Ziegler and Abel, 2014), dibenzyl ethoxymethylene malonate (DBEMM) (Rebane et al., 2015)) have been developed and applied for improving the analysis of amino acids.

Derivatization has also been applied for the analysis of peptides or proteins, but mainly for qualitative proteomics work (protein identification and characterization, de novo sequencing of proteins) and relative protein quantification (Qiao et al., 2014; Zhou et al., 2014). Recently, a novel selective peptide derivatization (SPD) strategy was developed for increasing sensitivity for the LC-MS/MS quantitative bioanalysis of proteins (Yuan et al., 2014b). This strategy is to selectively derivatize the surrogate peptide of the target protein while not reacting the background peptides. Therefore, the physicochemical differences between derivatized target peptides and underderivatized peptides would be enhanced, and their separation during extraction and chromatographic separation would be improved. In addition, the ionization efficiency, fragmentation pattern, and extraction recovery of the target peptide may increase significantly after it is derivatized, thus resulting in significantly enhanced assay sensitivity. To demonstrate the feasibility of the SPD strategy, a test mAb in monkey serum was first digested. The resulted tryptic digests were treated with malondialdehyde (MDA), a reagent that can selectively derivatize arginine containing peptides, and then purified by SPE. With the significantly decreased ion suppression and increased peptide extraction recovery after SPD treatment using MDA, a fivefold improvement in assay sensitivity was achieved. The SPD strategy was successfully applied in the development of an LC-MS/MS assay for the sensitive, accurate, and precise analysis of the test mAb in monkey serum.

#### 10.2.6 Online Sample Preparation

Conventional offline sample preparation typically involves multiple steps such as sample transfer, elution, drying down, and reconstitution. Loss of analyte could happen during these steps and result in reduced recovery and decreased sensitivity. For analytes that can be adsorbed (nonspecific binding) to the surface of the containers (e.g., sample vials, pipette tips, and 96-well plates), the loss during these steps can be even more

significant. Minimizing the loss of analyte during sample preparation is an important factor to consider for improving sensitivity, especially when developing a supersensitive assay. Online sample preparation is an effective approach for avoiding the sample loss that may happen during offline sample preparation steps. If so designed, it can greatly preconcentrate the analyte by injecting a large volume of sample into the loading column without sacrificing the chromatographic performance and therefore further improve the sensitivity. Its fully automated operation may also improve the quality and efficiency of the assay by avoiding manual operations and minimizing human errors. There has been increased application of online sample preparation such as online SPE (Kuklenyik et al., 2005; Clavijo et al., 2009; Jones et al., 2012; Kuklenyik et al., 2013; Rogeberg et al., 2014) and online IAE (Cai and Henion, 1996; Sen et al., 2003; Berna et al., 2006; Moser and Hage, 2010; Neubert et al., 2010; Dufield and Radabaugh, 2012). Column-switching approach using multiport valves is the most commonly used technique for online sample preparation. Typically, the sample is first injected into a loading column (could be an SPE column for online SPE or an immunoaffinity column for online IAE). After washing out the matrix components, the multiport valve is switched and the remaining extract is transferred (back or forward flushed) to an analytical column for LC separation and then MS analysis. Kuklenyik et al. (2005) developed an online SPE-LC-MS/MS method for analyzing trace levels of 18 perfluorinated organic acids and amides in the serum. Some of these chemicals (the volatile sulfonamide derivatives) could not be analyzed by a previously developed offline SPE method due to their vaporization during evaporation of the SPE extract. Using online SPE, the evaporation step was eliminated, and the recoveries of these compounds were significantly improved compared with the offline method. As a result, 18 analytes were measured with improved sensitivity using only one-fifth (200 µL instead of 1000 µL) of the serum used by the offline SPE method.

Peptides and proteins are well known for their tendency to stick to the surface of materials, and they often show greater adsorption than small molecule compounds (van Midwoud et al., 2007). Online IAE is an efficient method to mitigate this potential issue. In addition, it eliminates the time-consuming and labor-intensive offline immunocapture steps by combining them in a single online method. Therefore, it can achieve significantly improved sample preparation efficiency, as well as increased recovery and sensitivity. Online IAE has become a promising sample preparation strategy for the purification of peptides (Berna et al., 2006; Li et al., 2007; Neubert et al., 2010) and proteins (Hoos et al., 2006; Cingöz et al., 2010; Fan and Neubert, 2016).

### 10.3 LC Separation Strategies for Improving Sensitivity

Optimization of the LC method can enhance the signal intensity of the analyte or reduce the ion suppression, background noise, or interference from the matrix. As a result, significantly increased S/N and greatly improved sensitivity can be achieved for the assay. Column, mobile-phase modifiers or additives, and elution method are common parameters that can be optimized to achieve enhanced ionization efficiency and peak shape (more sharp and symmetric peak) for the analyte, therefore greatly improving its signal intensity. Various strategies (ultrahigh pressure liquid chromatography (UHPLC), 2D-LC, etc.) can also be used to improve the chromatographic separation of the analyte of interest from matrix components, therefore significantly reducing ion suppression and background noise.

#### 10.3.1 Optimization of Mobile Phase

The properties of the eluent (e.g., pH, nature and concentration of mobile-phase modifiers or additives, organic–aqueous solvent ratio) have a significant effect on the ionization of analytes and need to be carefully evaluated to achieve the best sensitivity. Usually, the presence of an acid (e.g., formic acid or acetic acid) in the mobile phase can promote the protonation of the analytes with basic functional groups (e.g. amines) and therefore enhance their ionization in positive ion mode. Similarly, a basic mobile phase will facilitate the deprotonation of the acidic analytes (e.g., carboxylic acids) and improve their ionization in negative ion mode. It is worth pointing out that in many cases, wrong-way-round ionization is observed in ESI: protonated analytes can be readily produced under basic conditions and deprotonated analytes under acidic conditions. For example, Wu et al. (2004) investigated the effects of various mobile-phase modifiers, including acids, neutral salts, and bases, on the responses of four selective androgen receptor modulators in ESI negative mode. Acetic, propionic, and butyric acids in the concentration range of 10 µM to 10 mM were found to improve the responses of the analytes, with the maximum improvement observed at a concentration of 1 mM. No favorable effects on the responses of the analytes in ESI negative mode were observed for neutral salts (ammonium formate, ammonium acetate), bases (ammonium hydroxide, triethylamine), and formic acid under most conditions. Peng and Farkas (2008) compared the responses of a variety of basic drugs (more than 45) covering a wide range of hydrophobicity ( $\log P$  0.09–7.6) and basicity ( $pK_a$  6.8–10) in ESI positive mode. Better MS responses and detection limits were achieved in high-pH basic mobile phases

than those in acidic mobile phases for most of the tested compounds in ESI positive mode. A similar phenomenon was also observed by Rainville et al. (2012). They compared basic (pH 10) and acidic (pH 3) aqueous mobile phases for the analysis of 24 probe pharmaceuticals. They found that increase in responses (up to 9.6-fold) was observed in 22 out of the 24 compounds tested when using basic mobile phase in ESI positive mode.

The MS responses of analytes in ESI usually increase with higher percentages of organic solvent in the eluent. Adjusting the mobile phase composition, column stationary phase, or LC elution method can change the retention of an analyte on the stationary phase and, therefore, enable the elution of the analyte in higher content of organic solvent and improve its MS response and sensitivity. For example, polar compounds often elute at low percentage of organic solvent in RP chromatography, resulting in poor sensitivity. Hydrophilic interaction liquid chromatography (HILIC), with its use of high percentages of volatile organic solvents in the mobile phase, can provide greatly improved sensitivity and has been widely used the analysis of polar compounds (Jian et al., 2010). Post-column addition of organic solvent or modifier is another useful approach to improve the MS response of the analyte. Mawhinney et al. (2007) reported that post-column addition of aprotic solvents (e.g., acetonitrile, ethyl acetate, 1,4-dioxane) and longer-chain alcohols (e.g., 1-propanol) significantly enhanced the responses of alkyl methylphosphonic acids in ESI negative mode and achieved enhancement of up to 60-fold for the signal intensity and up to 19-fold for the S/N of the chromatographic peaks. As shown in Figure 10.2, the post-column addition of acetonitrile not only significantly improved the peak intensity of 2-methylpropyl methyl phosphonic acid but also resulted in a 19-fold improvement of the S/N.

### 10.3.2 2D-LC

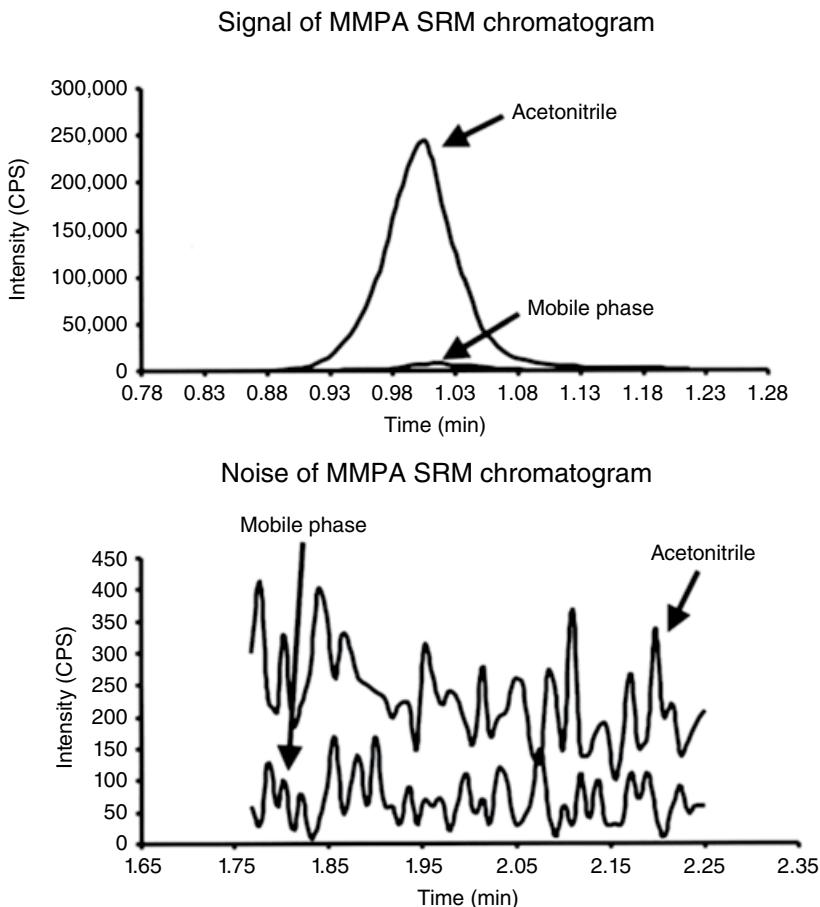
2D-LC, which utilizes two different LC separation of the sample, has a greatly improved resolving power compared to one-dimensional LC (Stoll, 2015). It can significantly enhance the chromatographic separation and reduce the sample complexity, therefore achieving much reduced matrix effect and background noise and increased sensitivity. Commonly used 2D-LC separation approaches include different combinations of RP (high pH, low pH, various stationary phases), HILIC, and ion exchange LC methods. Ideally, to maximize the resolving power, the separation mechanisms in the two LC dimensions should be completely orthogonal or independent. For quantitative bioanalytical methods, the most commonly used 2D-LC separation mode is heart-cutting (LC-LC), which works by transferring only the eluent

portion containing the analytes from the first dimension to the second dimension. One major challenge for 2D-LC is that it requires careful, and often complicated, optimization of the LC methods in both dimensions.

2D-LC has been applied for achieving highly sensitive bioanalysis of various analytes, including small molecule compounds (Li et al., 2011b; Chi et al., 2016), peptides (Halquist et al., 2012; Chambers et al., 2014), and proteins (Berna et al., 2007; Xu et al., 2010; Shen et al., 2015). Chi et al. (2016) developed an ultrasensitive method for the quantification of tiotropium bromide in human plasma by 2D-LC-MS/MS. Using a combination of a cyano column and a C18 column for the 2D-LC separation, they obtained an LLOQ of 0.100 pg/mL for tiotropium with a S/N > 10, which was at least fivefold better compared with the 1D-LC method. Due to its high resolving power, 2D-LC is particularly suitable for achieving highly sensitive analysis of complex samples (e.g., tryptic digests of plasma/serum samples). Shen et al. (2015) developed and validated an online 2D-LC-MS/MS method for quantifying a therapeutic protein immunoglobulin A1 protease in human serum. They used a high pH (pH 8) mobile-phase RP separation in the first dimension and a low pH (pH 3) RP separation in the second dimension to achieve the orthogonal chromatographic separation of the samples. As shown in Figure 10.3, for the sample analyzed by the 1D-LC method, the surrogate peptide peak was severely affected by the interference peaks and high background noise, making it hard to integrate for quantification purposes. In contrast, for the sample analyzed by the 2D-LC method, much better chromatographic separation, higher peak intensity, and lower background were achieved. As a result, the online 2D-LC method achieved an LLOQ of 0.05 µg/mL, which was more than 40-fold improvement in sensitivity compared with the 1D-LC method.

### 10.3.3 Low-Flow LC

In recent years, low-flow LC or miniaturized LC (e.g., microflow LC, nanoflow LC) has drawn considerable attention and interest for improving the sensitivity of LC-MS assays (Duan et al., 2010; Neubert et al., 2012; Percy et al., 2012; Christianson et al., 2013; Olkowicz et al., 2015; Wilffert et al., 2016). Low-flow LC uses columns with much smaller internal diameter (ID) (e.g., <1 mm for microflow LC and <0.150 mm for nanoflow LC) and hence operates at a much lower volumetric flow rate compared to regular-flow LC with ID of 2.1 mm or above. In such analyses, “low flow” refers to the volumetric flow rate. However, the linear flow velocity is relatively similar to “regular” HPLC in order to maintain appropriate separation kinetics. Low-flow LC requires



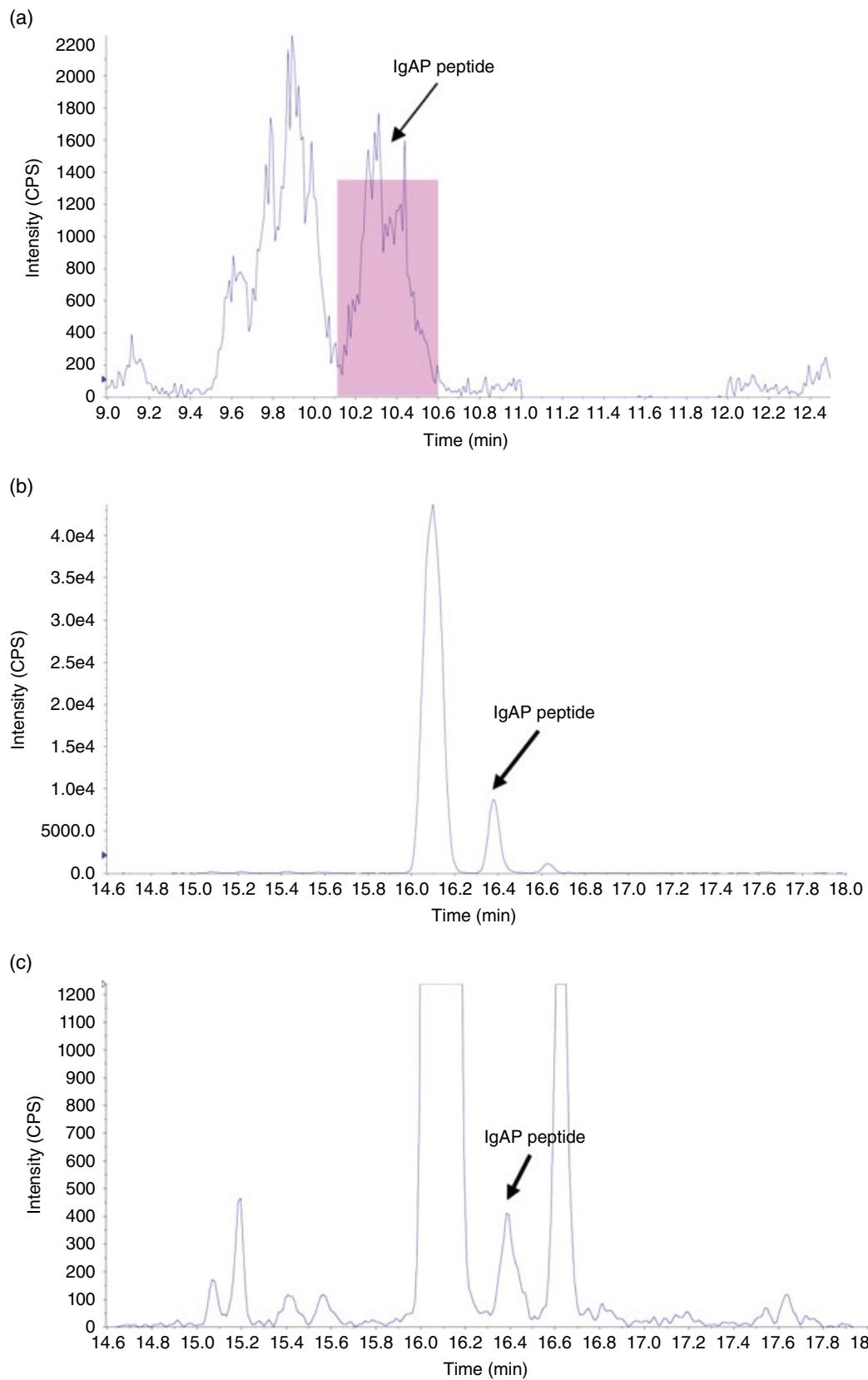
**Figure 10.2** Comparison of signal and signal-to-noise (S/N) regions of the SRM chromatogram of MMPA with the post-column addition of acetonitrile. MMPA, 2-methylpropyl methyl (13CD3) phosphonic acid. Source: Mawhinney et al. (2007). Reproduced with permission of Springer.

less sample injection volume to achieve a similar or better sensitivity compared to regular LC, making it particularly suitable for achieving highly sensitive analysis of samples with a limited volume. Another advantage of low-flow LC is its much reduced consumption of mobile phase.

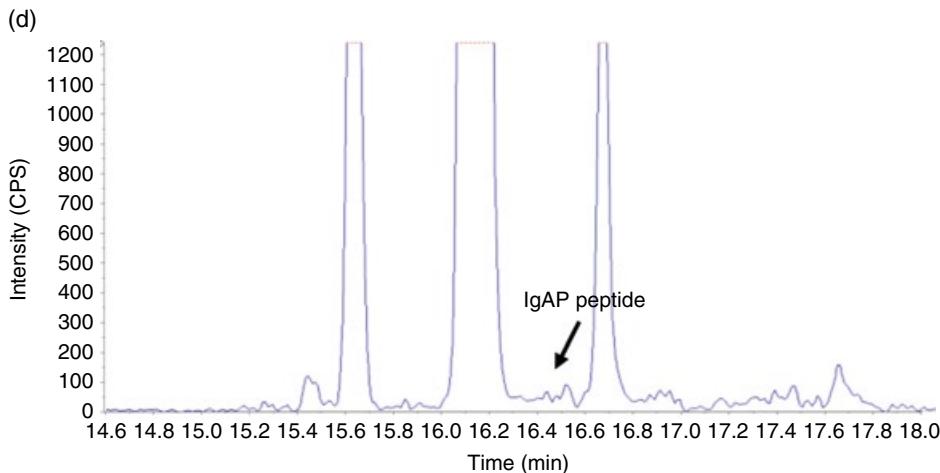
Several characteristics of low-flow LC contribute to the sensitivity improvement of LC–MS assays. In cases where the available sample is limited, low-flow LC systems offer an advantage. During the chromatographic separation process, the concentration of the analyte will be diluted, and this chromatographic dilution (the ratio of the original analyte concentration at the head of the column to the final analyte concentration at the end of the column) is proportional to the square of the column radius (Vissers et al., 1997). As a result, for a given injected volume, using a smaller ID column will have much less chromatographic dilution and can result in a significantly higher concentration of the analyte after chromatographic separation. For example, with a fixed injection volume, a decrease in the ID of a column from 4.6 to 0.300 mm will result in a 235-fold increase in concentration at the end of the column. It must be emphasized that this sensitivity improvement can only be fully realized when the same volume of sample can be

loaded onto the column. However, in order to maintain chromatographic performance, as the column dimension is scaled down, the injected volume also has to be scaled down; thus the smaller loading capacity of low-flow LC often offsets its sensitivity gain. An online trapping system (e.g., a column-switching device) can be used for the injection of a larger volume of sample (Kataoka and Saito, 2012), therefore maximizing the ability of low-flow LC in increasing sensitivity. The decrease in flow rate (for the LC flow delivered to the MS) usually results in decreased droplet size and enhanced desolvation, thus increasing ionization and ion transfer efficiency of the analyte in ESI (Wilm and Mann, 1996; Tang et al., 2004). In addition, the decreased ion suppression at very low flow rate can contribute to the improved sensitivity, since the smaller droplet often results in a decreased number of compounds per droplet and reduced competition for charges during ionization. Ion suppression to the test compounds was found to be completely absent at flow rates below 20 nL/min (Schmidt et al., 2003).

Downscaling column ID from microflow LC to nanoflow LC can achieve additional gain in sensitivity. D’Orazio et al. (2012) compared nanoflow LC (50 µm ID column) and microflow LC (1.0 mm ID column) using steroids and nonsteroidal anti-inflammatory drugs



**Figure 10.3** (a) SRM ion chromatogram of the spike-in surrogate peptide in serum tryptic peptides (equivalent to 2.0  $\mu\text{g/mL}$  IgAP) separated by the first dimension RPLC system; (b) SRM ion chromatogram of the surrogate peptide at the same concentration following separation by the 2D-LC system (the highlighted fraction in (a) was transferred to the second RP column); (c) SRM ion chromatogram of the spike-in surrogate peptide at 0.05  $\mu\text{g/mL}$  following separation by the 2D-LC system; (d) surrogate peptide SRM ion chromatogram of the blank matrix following separation by the 2D-LC system. *Source:* Shen et al. (2015). Reproduced with permission of American Chemical Society.



**Figure 10.3** (Continued)

as model compounds. They used the same linear flow velocity and isocratic mobile phases for the two LC systems. The injection volume was also optimized to allow maximal loading of the sample onto the column without compromising the chromatographic performance. The nanoflow LC system ( $3.6\text{ }\mu\text{L}$  injection volume) achieved approximately 10-fold better detection limit and approximately fivefold better LLOQ than the microflow LC system ( $41.6\text{ }\mu\text{L}$  injection volume) using a UV detector. Olkowicz et al. (2015) compared nanoflow LC ( $100 \times 75\text{ }\mu\text{m}$  ID) and microflow LC ( $100 \times 1\text{ mm}$  ID) for the LC–MS/MS quantification of cardiac troponin T, a protein biomarker, in mouse hearts. The nanoflow LC method achieved fivefold improvement in sensitivity compared with the microflow LC method with a less injection volume ( $0.5\text{ }\mu\text{L}$  vs.  $2.5\text{ }\mu\text{L}$ ), although this comparison was not a direct head-to-head comparison (since the two LC methods had different linear flow velocities and gradient slopes). By using an online trapping system to preconcentrate the sample, they achieved additional gain in sensitivity, but with the cost of a much longer run time. One potential issue for nanoflow LC is the technical challenge to achieve reproducible and robust analysis, which needs to be considered and carefully evaluated for large-scale application.

The cleanliness of the extracted sample is an important factor for the effective utilization of the advantage of low-flow LC, considering its limited loading capacity. Efficient sample purification and enrichment can ensure the realization the sensitivity gain on low-flow LC (Wilffert et al., 2016). In one study, low-flow LC showed worse sensitivity compared with the standard-flow LC platform (Percy et al., 2012), which may due to the insufficient sample cleanup.

## 10.4 MS Detection Strategies for Improving Sensitivity

Tremendous improvements in mass spectrometer instrumentation have been achieved in the past two decades. SRM-based LC–MS/MS detection using triple quadrupole mass spectrometers is the most commonly used approach for quantitative analysis. Newer generations of triple quadrupole instruments can easily deliver more than 10 times better sensitivity than old models (e.g., a Triple Quad 6500 vs. an API4000). Advancements in other types of instrumentation (e.g., high-resolution mass spectrometry (HRMS) and ion mobility spectrometry (IMS)) provide additional options and strategies for improving sensitivity.

### 10.4.1 SRM

LC–MS/MS using SRM, because of its excellent selectivity and sensitivity, is the gold standard for quantification of small molecule analytes. SRM is also widely used for the quantification of peptides and proteins. Careful optimization of the SRM parameters is critical to maximize MS detection sensitivity. For MS optimization, the first step is to choose an appropriate ionization mode. ESI is the most commonly used ionization mode and is often used as the starting point for optimizing the MS detection of the analyte of interest. For compounds that do not ionize well in ESI (in either positive or negative mode), other ionization modes, such as atmospheric pressure chemical ionization (APCI), can be evaluated. For example, for nonpolar compounds (e.g., steroids, polycyclic aromatic hydrocarbons), APCI often provides better ionization and sensitivity than ESI. In addition, APCI

usually is less affected by matrix effect or buffer/additives used in the mobile phases compared with ESI, and these can be utilized for improving sensitivity in some cases. However, APCI is not as soft as ESI and it operates at high temperatures; therefore, it cannot be used for thermally unstable compounds and it potentially generates more in-source degradation of analytes. After the ionization mode is selected, the SRM transitions covering the major product ions of the analyte are compared. Typically, the most intense SRM transition will be selected for the MS detection of the analyte. It is noteworthy that the matrix components in the sample extracts may cause different matrix effect or even interference to individual SRM transitions, especially in complex samples (e.g., digested biological samples for the analysis of proteins/peptides). Thus, the most sensitive SRM transition identified using a neat standard solution may undergo more severe matrix effect and become less sensitive than other transitions in the extracted biological samples. The optimized MS method, if possible, needs to be evaluated and verified using extracted biological matrix.

The MS parameter optimization for peptides and proteins is much more complicated than that for small molecules. Ionization of a small molecule typically results in a predominant singly charged ion, and its fragmentation to the most abundant product ion is used for the sensitive SRM detection of the analyte. On the other hand, peptides and proteins usually have ions distributed over multiple charge states. The fragmentation pattern and the intensities of the product ions generated from the peptide ions under different charge states may vary significantly (Zhang et al., 2011). Thus, the most abundant ion identified in the first quadrupole (Q1) may not generate the most abundant product ion, and the corresponding SRM transition may not be the most sensitive one. The charge state distribution could be modified by changing mobile-phase additives (Iavarone et al., 2000; Miladinović et al., 2012) or even MS parameters (e.g., declustering potential) (Zhang et al., 2011). In addition, the endogenous components in the biological matrix may cause different matrix effect to various SRM transitions. Therefore, to obtain the best sensitivity, the SRM transitions from major precursor ions at various charge states need to be carefully evaluated and compared, preferably, using sample extracts from the target biological matrix.

Using the adduct ion of the analyte as the precursor ion in SRM has been applied for increasing the MS responses in both positive (Zhao et al., 2002; Mortier et al., 2004) and negative (Gu et al., 2010; Ji et al., 2015) ion mode. The protonated ion (positive mode) or the deprotonated ion (negative mode) of the analyte is

usually the most abundant ion formed during ionization and selected as the precursor ion. Additives or modifiers in the mobile phase could affect the distribution of the analyte among protonated (or deprotonated) ion and various adduct ions (e.g., ammonium, sodium, and potassium adduct ion in positive mode and formate and acetate adduct ion in negative mode). Sometimes, one type of adduct ion can be the predominantly formed ion and can be used to improve sensitivity. For example, to improve the sensitivity for the analysis of dapagliflozin, Ji et al. (2015) evaluated the formation of adduct ions in the presence of various additives (formic acid, acetic acid, ammonium formate, ammonium acetate, ammonium hydroxide) in the mobile phases. They found that when ammonium acetate or acetic acid was in the mobile phases, the acetate adduct ion became the dominant ion in negative ESI mode. Using the acetate adduct ion as the precursor ion for the SRM detection of dapagliflozin, they obtained a fivefold improvement of the assay sensitivity (LLOQ at 0.2 ng/mL with 50 µL of plasma).

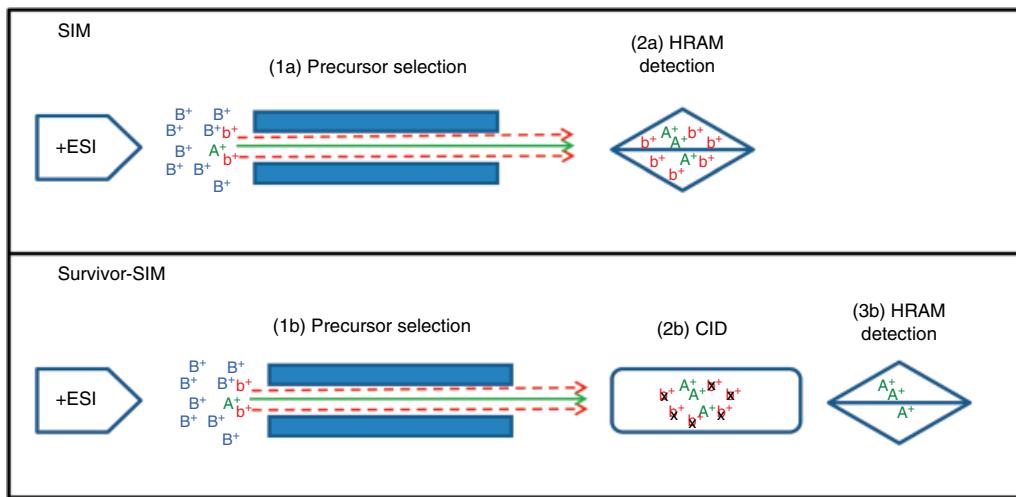
Summation of different SRMs from the same analyte is also a useful approach to increase sensitivity in LC-MS/MS methods (Nitin et al., 2003; Manjunath Swamy et al., 2010; Li et al., 2016). Manjunath Swamy et al. (2010) evaluated the use of this summation approach for the LC-MS/MS analysis of clopidogrel and ramiprilat and achieved greater than twofold improvement of sensitivity for both compounds compared to the single SRM transition methods. Similarly, Li et al. (2016) applied a summation of five SRM transitions for the quantification of catecholamines in human peripheral blood mononuclear cells and achieved three- to fourfold improvement of sensitivity. Theoretically, this approach should only work for analytes having multiple abundant product ions. For estradiol, a compound with only one major product ion, Pauwels et al. (2013) used a summation of five identical SRM transitions and still achieved a fourfold (300%) increase of signal and 1.5-fold (50%) increase of S/N. This may be because that the random noise peaks of the five SRM mass spectra were mostly not correlated; therefore, the increase in the noise was less than the increase in the signal, and the S/N increased. This summation approach could be very useful for improving sensitivity for the analysis of peptides or proteins. The spreading of ions over multiple charge states in the ionization of peptides or proteins often results in lower ion intensities in individual charge state and thus poor assay sensitivity. The summation of multiple SRM transitions from various charge states could significantly improve the sensitivity. Yet, caution needs to be taken to ensure the selectivity of the assay when using the summation.

### 10.4.2 High-Resolution Mass Spectrometry (HRMS)

HRMS instruments have achieved tremendous improvements in sensitivity, resolution, scan speed, and dynamic range in recent years. The detection limit of the newest generation of HRMS instruments, such as q-TOF and Orbitrap, is approaching what triple quadrupole instruments can achieve. HRMS can also obtain quantitative and qualitative information in the same analysis. Consequently, HRMS has gained tremendous interest and attention as an alternative technique to SRM for quantitative analysis. There are two major MS acquisition approaches for HRMS: the nontargeted approach by monitoring all ions over a mass range and the targeted approach by acquiring specific ions or the product ions of specific precursor ions. Usually the targeted approach can achieve better sensitivity compared with the nontargeted approach. Nevertheless, the nontargeted approach allows post-acquisition data mining to generate qualitative information in addition to the quantitative data, which is a unique and attractive feature for HRMS. Compared with SRM detection using a triple quadrupole instrument, HRMS was able to achieve better selectivity (Kaufmann et al., 2010; Fedorova et al., 2013; Hamelin et al., 2013; Morin et al., 2013) and comparable or even better sensitivity (Ramanathan et al., 2011; Dillen et al., 2012; Hamelin et al., 2013; Morin et al., 2013) for the quantitative analysis of both small and large molecules.

HRMS has been used as a MS detection strategy for improving sensitivity. It is particularly suitable for the following two types of applications. HRMS works very well for samples that have severe interference (e.g., isobaric compounds) to the analyte during LC-MS analysis. The interfering compound has precursor and

product ion with the same nominal mass as the analyte of interest and, thus, cannot be differentiated by SRM detection using a unit resolution triple quadrupole instrument. Utilizing the high selectivity (resolution) of HRMS, compounds with very small mass differences can be differentiated, the interference can be eliminated, and, therefore, the assay sensitivity can be greatly improved (Wei et al., 2012; Bowen et al., 2016). HRMS can also achieve superb sensitivity for molecules exhibiting poor fragmentation (Morin et al., 2013) or molecules resistant to collision-induced dissociation (CID) fragmentation (Ciccimaro et al., 2014). For instance, for the analysis of somatostatin, a cyclic peptide with poor fragmentation, HRMS monitoring of the parent-to-parent transition ( $m/z$  546.5  $\rightarrow$  546.5 using a TripleTOF 5600) achieved two- to threefold increase in S/N compared to the SRM analysis using a triple quadrupole instrument, since this HRMS approach minimized the sensitivity loss during fragmentation (Morin et al., 2013). Ciccimaro et al. (2014) applied a survivor-selected ion monitoring (survivor-SIM) approach for achieving improved sensitivity for cyclic peptides with poor fragmentation. As illustrated in Figure 10.4, by applying a collision energy that can break down the isobaric background ions but not the target analyte ions in CID, HRMS survivor-SIM can efficiently remove the background ions and gain additional selectivity and therefore can achieve improved sensitivity compared with HRMS SIM analysis. For the CID-resistant cyclic peptides they evaluated, approximately 50-fold improvement in assay performance was achieved by switching from SRM (using a triple quadrupole MS) to HRMS SIM (using an Orbitrap instrument). Using HRMS survivor-SIM, a five- to tenfold improvement in LLOQ



**Figure 10.4** HRMS SIM and survivor-SIM showing simplified experimental events. Source: Ciccimaro et al. (2014). Reproduced with permission of American Chemical Society.

was achieved compared with HRMS SIM in extracted plasma samples. To further improve sensitivity, ions from the most intense charge states and/or isotopes of the analyte can be summed for the quantification (Ramagiri and Garofolo, 2012). However, to ensure the selectivity of the assay, the charge states or the isotopic peaks used for the summation must be carefully evaluated using extracted blank matrix to avoid interference from endogenous components in the sample matrix.

#### 10.4.3 IMS

IMS is an analytical technique that can separate ions based on their mobility in the gas phase in a carrier buffer gas and an electrical field. The mobility of an ion is determined by a combination of the ion's mass, charge, size, and shape, and not by its mass-to-charge ratio as in regular MS; in this way, it is more analogous to free-solution electrophoresis than to MS. IMS can provide an additional dimension of selectivity and, therefore, may separate the interfering components in the sample and achieve improved sensitivity. Fu et al. (2016) used differential mobility spectrometry (DMS), a recently arisen IMS technology, in combination with SIM for the quantification of pasireotide (a therapeutic cyclic peptide with poor fragmentation) in human plasma. By adding DMS to SIM, the background noise was significantly reduced, and the assay sensitivity was improved

approximately fivefold compared with that obtained in SRM or SIM without DMS. The developed LC-DMS-SIM method was validated with good specificity and ruggedness over the range of 0.01–50 ng/mL using 0.1 mL of human plasma.

## 10.5 Conclusions

Improving assay sensitivity is often challenging, yet critical, for quantitative bioanalysis of biomarker and other molecules. The strategies described previously cover the three major interconnected components of an LC-MS bioanalytical assay: sample preparation, LC separation, and MS detection. Application of a single strategy or optimization of one component may not be enough to achieve the desired sensitivity. Obtaining a supersensitive assay usually requires a systematic method development approach that can incorporate various strategies and achieve overall optimized conditions for all the three components.

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# 11

## Strategies to Improve Specificity for Targeted Biomarker Quantitation by LC–MS

*Yuan-Qing Xia and Jeffrey D. Miller*

*Sciex, Framingham, MA, USA*

### 11.1 Introduction

Robustness of liquid chromatography–mass spectrometry (LC–MS) bioanalytical methods is critical in providing support for quantitative determination of drug candidates, metabolites, and endogenous biomarkers from biological matrices (Jemal and Xia, 2006; Jemal et al., 2010). One of the most important parameters of method robustness is the assay specificity, which ensures that the assay measures accurately the intended analytes without any interferences from other components. Similar to drug candidates and their metabolites, sensitive and specific measurement is also essential to the biomarker quantitation. Unlike drug candidates or their metabolites for which blank matrices without the presence of analytes can be easily available, the very nature of endogenous biomarkers dictates that the target analytes are always present in the biological matrices. Thus, even with the superior specificity of mass spectrometers, one can always question whether or not the peak at the retention and mass spectrometric ion transitions of the authentic biomarker standard is indeed the targeted analyte. Inadequate specificity can also lead to elevated baseline and thus poor assay sensitivity. Additionally, poor specificity due to insufficient chromatographic resolution of analyte of interest and matrix components such as phospholipids or dosing vehicles can lead to severe matrix effect due to ionization suppression. In the present chapter, we would like to discuss specificity enhancement in mass spectrometric detection in LC–MS bioanalysis. In particular, we would like to focus our discussion on differential mobility spectrometry (DMS) for reducing high chemical background noise, eliminating matrix interferences, and separating isobaric metabolites in quantitative LC–MS bioanalysis. Furthermore, we would like to illustrate strategies for enhancing the mass spectrometric specificity by utilizing high-resolution mass spectrometry (HRMS) in the

LC–MS bioanalysis. These are two relatively new advances in quantitative bioanalysis that have been successfully used more extensively in target drug bioanalysis, and the trend increases to incorporate them in biomarker analysis. Besides DMS and HRMS, other traditional approaches of enhancing assay specificity such as column-switch technology, two-dimensional LC, derivatization, and orthogonal analysis such as comparing the data from LC–MS with ELISA may also be used when DMS or HRMS is unavailable. However, discussion on those applications is beyond the scope of the current chapter.

### 11.2 Differential Mobility Spectrometry

Due to its superb sensitivity and specificity, LC–MS, and in particular LC in conjunction with tandem triple quadrupole mass spectrometers (LC–MS/MS), has been the method of choice in quantitative bioanalysis of numerous molecules from biological matrices. Most commonly, analysts use LC–MS/MS as the primary platform for quantitation assays, employing multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) experiments to achieve their goals. An important basic requirement in developing a selective and sensitive MRM method on a triple quadrupole mass spectrometer is to obtain one major or multiple abundant fragment ion(s) via collision-induced dissociation (CID). However, it may prove a very challenging task to find such CID fragment ions for building an MRM method for some analytes, such as cyclic peptides that are usually resistant to the CID fragmentation due to their knotted tertiary structure and nonmobile proton(s) (Ciccimaro et al., 2014). These types of analytes may resist CID fragmentation at lower collisional energies or, with high collision energy conditions, generate only nonspecific small

immonium ions that are not suitable for specific and sensitive MRM analysis. An alternative approach to manage the issue is to apply selected ion monitoring (SIM) to select the intact precursor ion in Q1 mass analyzer, which overcomes the issues of deficient CID fragmentation while maintaining the analyte signal intensity. However, this method has some significant drawback such as a high background baseline from biological matrices. Under unit resolution in triple quadrupole mass spectrometers, numerous interfering ions that share the same  $m/z$  values as the analyte can also reach the MS detector, leading to a reduced assay specificity and nonoptimal signal-to-noise (S/N) as compared with a typical MRM method. Another intact precursor selection method is to use multiple ion monitoring (MIM) or parent-to-parent pseudo-MRM for addressing analytes with inefficiency CID fragmentation (Ceccato et al., 2003). In this approach, the most abundant precursor ion is selected in the Q1 of a triple quadrupole mass spectrometer. By optimizing collision energy to minimize fragmentation of the analyte and maximize the fragmentation of the isobaric matrix components, the precursor ion of the analyte passes through the collision cell with a minimum intensity loss, while the isobaric matrix components are eliminated by fragmenting in the collision cell. Then, the abundant intact precursor ion for the analyte of interest passes through Q3 to the detector. This approach sometimes also faces significant challenges in matrix interference and/or elevating background baseline that limits the applicability of the methodology (Fu et al., 2016; Xia et al., 2017).

High-field asymmetric waveform ion mobility spectrometry (FAIMS) or DMS has emerged in recent years as a powerful separation technique for isobaric

compounds and other moieties that are difficult to be resolved by traditional chromatographic methods or specific mass-to-charge transitions (Shvartsburg et al., 2006; Coy et al., 2010; Schneider et al., 2010; Lapthorn et al., 2013; Campbell et al., 2014). Figure 11.1 shows a schematic overview of the DMS coupled with a Sciex QTRAP 5500 system mass spectrometer. DMS, when used in conjunction with LC-MS/MS, acts as a post-column and pre-mass spectrometer ion filter in which only selected ions generated from electrospray ionization (ESI) sources are transmitted. In brief, DMS operates with an asymmetric electric field applied between two planar electrodes perpendicular to the ion motion. The ion mobility of analyte ion changes as it experiences the pulsed low- and high-field segments of the oscillating electric field. Ions with a particular mobility can be guided through the device by applying a compound-specific compensation voltage (COV) in combination with a characteristic separation voltage (SV). By using DMS, the chemical background can be significantly reduced or eliminated (Klaassen et al., 2009; Schneider et al., 2010). This can be reflected by a significant increase in S/N and assay specificity. With incorporation of certain modifiers, the efficiency of DMS in separating analyte of interest from interfering components can be further enhanced (Schneider et al., 2010).

DMS has been applied to improve assay specificity for analysis of steroids. Challenges for steroid analysis by LC-MS/MS include low ionization efficiency, endogenous isobars with similar fragmentation patterns, and chromatographic retention. AB Sciex 5500 mass spectrometer equipped with DMS provides an additional degree of separation prior to MS/MS detection and shows promise in improving specificity of analysis (Ray et al.,

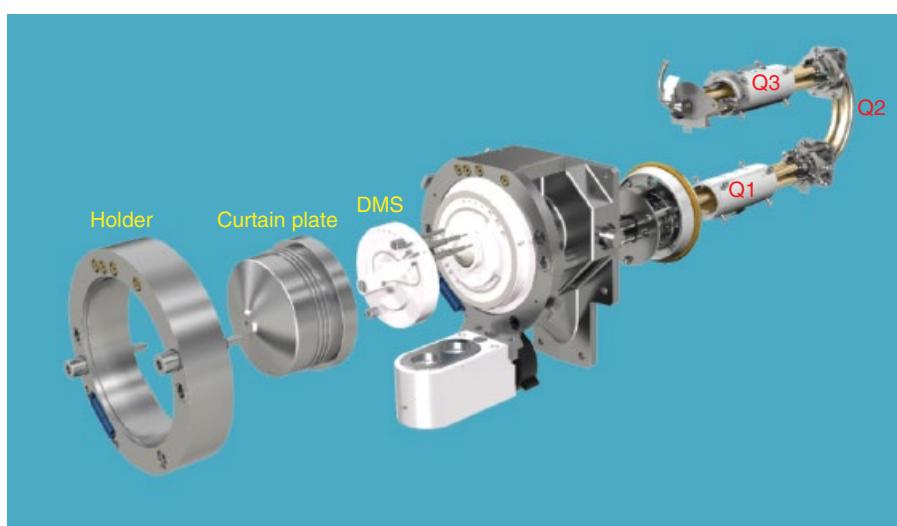


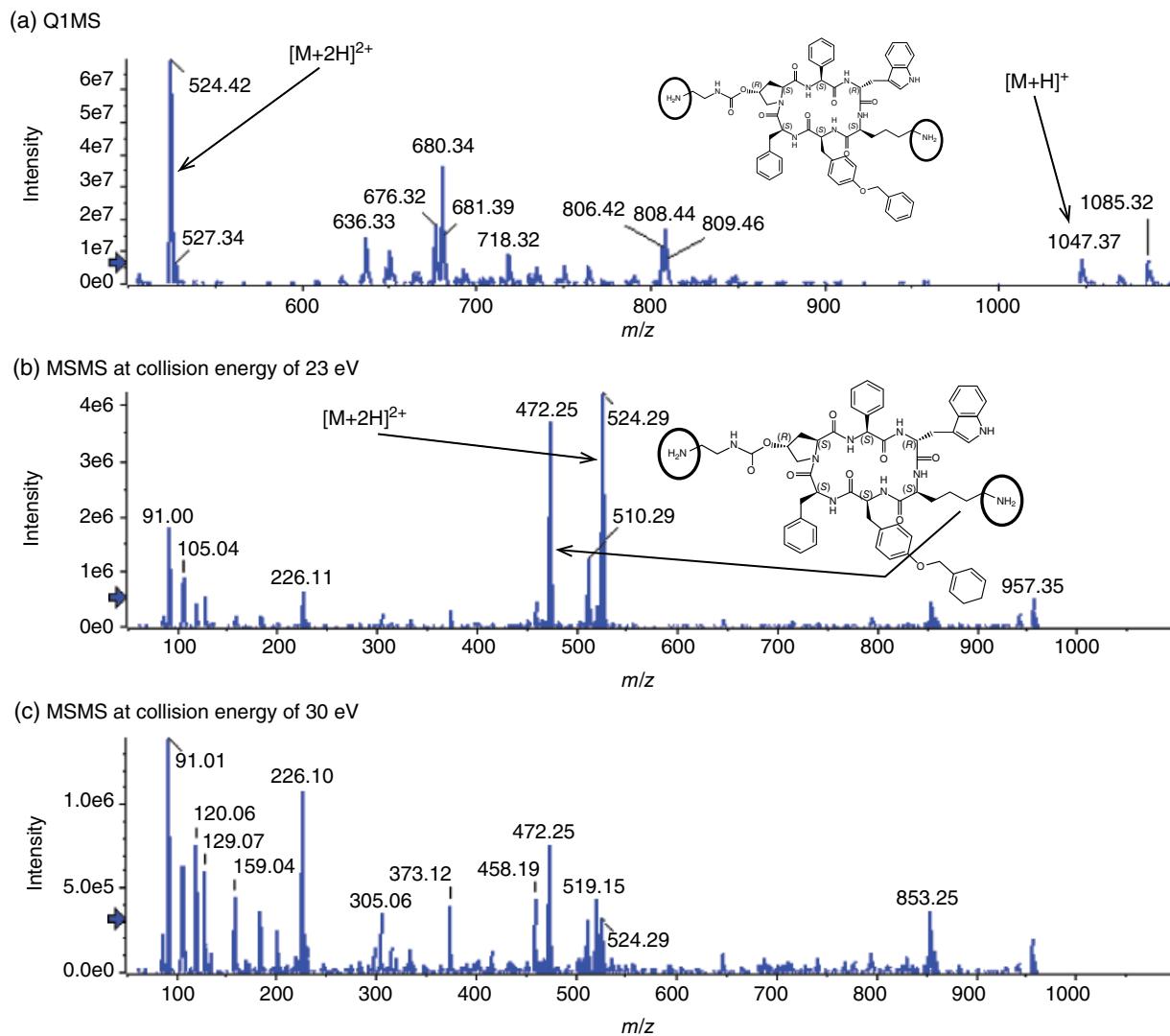
Figure 11.1 A differential mobility spectrometry coupled to a QTRAP 5500 system.

2015). An LC-DMS-MS/MS method was developed for analyzing endogenous biomarkers corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17-hydroxyprogesterone, and progesterone in human serum and plasma that has acceptable performance characteristics for diagnostic applications. By the effective combination of LC and DMS, the background noise was reduced, and the isomeric pairs of analytes were successfully separated. Another endogenous biomarker, testosterone, was also analyzed by LC-DMS-MS/MS with improved specificity and sensitivity while using simple sample preparation via acetonitrile precipitation of serum samples (Woroniecki et al., 2012). With the conventional LC-MS/MS method, both MRM transitions of  $m/z$  289 to  $m/z$  109 and  $m/z$  289 to  $m/z$  97 showed significant endogenous matrix interference peaks co-eluting with testosterone, resulting in elevated baseline and reduced specificity and sensitivity. When applying DMS, the endogenous matrix interference peaks were eliminated, and the LC-MS/MS chromatograms show an excellent analyte peak with very clean baseline, making peak integration much easier for quantitation of testosterone. Use of DMS allows simplification of sample preparation, and achievement of high specificity and sensitivity of analysis. Specificity gained with DMS allows reducing the complexity of sample preparation, decreasing LC run times, and increasing the speed of the analysis.

DMS is also successfully used to analyze other types of compounds such as peptides. Due to their good therapeutic efficacy and low toxicity as well as possibility as useful endogenous biomarkers for various biochemistry pathways, great interest has been recently paid to analyze either endogenous or modified peptides in biological samples (Cox et al., 2016). For the analysis of peptides with inefficient CID fragmentation, a reported method using FAIMS coupled with pseudo-MRM in the analysis of a peptide on a triple quadrupole MS demonstrated the significant improvement of assay sensitivity in comparison with conventional pseudo-MRM (Klaassen et al., 2009). On the other hand, Brown et al. (2012) successfully employed FAIMS in combination with in-source CID fragmentation technique for the time-of-flight mass spectrometry (TOFMS) analysis to efficiently remove the PEG interference ions from pharmaceutical excipients, leading to improvement of the bioanalytical assay selectivity. Accordingly, addition of a DMS device to a conventional triple quadrupole or QTRAP mass spectrometer can be the method of choice in LC-MS/MS combined with pseudo-MRM or MIM for quantitative analysis of molecules with inefficiency CID fragmentation. Xia successfully exploited LC-DMS-pseudo-MRM workflow in quantitative bioanalysis of sunflower trypsin inhibitor (SFTI) in rat plasma (Xia et al., 2015, 2017). SFTI, cyclo-GRCTKSIPPICFPD, is a 14-amino acid

disulfide-bonded peptide that possesses exceptionally potent trypsin inhibitory activity and has promise as a stable peptide-based drug template. It combines a head-to-tail cyclized backbone and a disulfide bond so that it is difficult to be fragmented to generate specific and abundant fragment ions to be used in MRM method under CID conditions. In order to address the challenge, a pseudo-MRM method was initially employed with the same doubly charged precursor ion of  $m/z$  757 selected in both Q1 and Q3. However, results from the pseudo-MRM analysis displayed a high background baseline around the elution time of the analyte, impacting a desirable lower limit of quantitation (LLOQ). A DMS method was developed to address the challenge of the high matrix baseline while retaining the analyte signal intensity. The method selected the intact precursor ion in Q1/Q3 mass analyzers to maintain the analyst signal and used DMS to reduce matrix baseline interference. The method showed a 40-fold assay sensitivity improvement over the pseudo-MRM method without using DMS. In addition to substantial improvement in sensitivity, the DMS-pseudo-MRM significantly contributed to the excellent assay accuracy and precision achieved for the quantitation of SFTI in rat plasma.

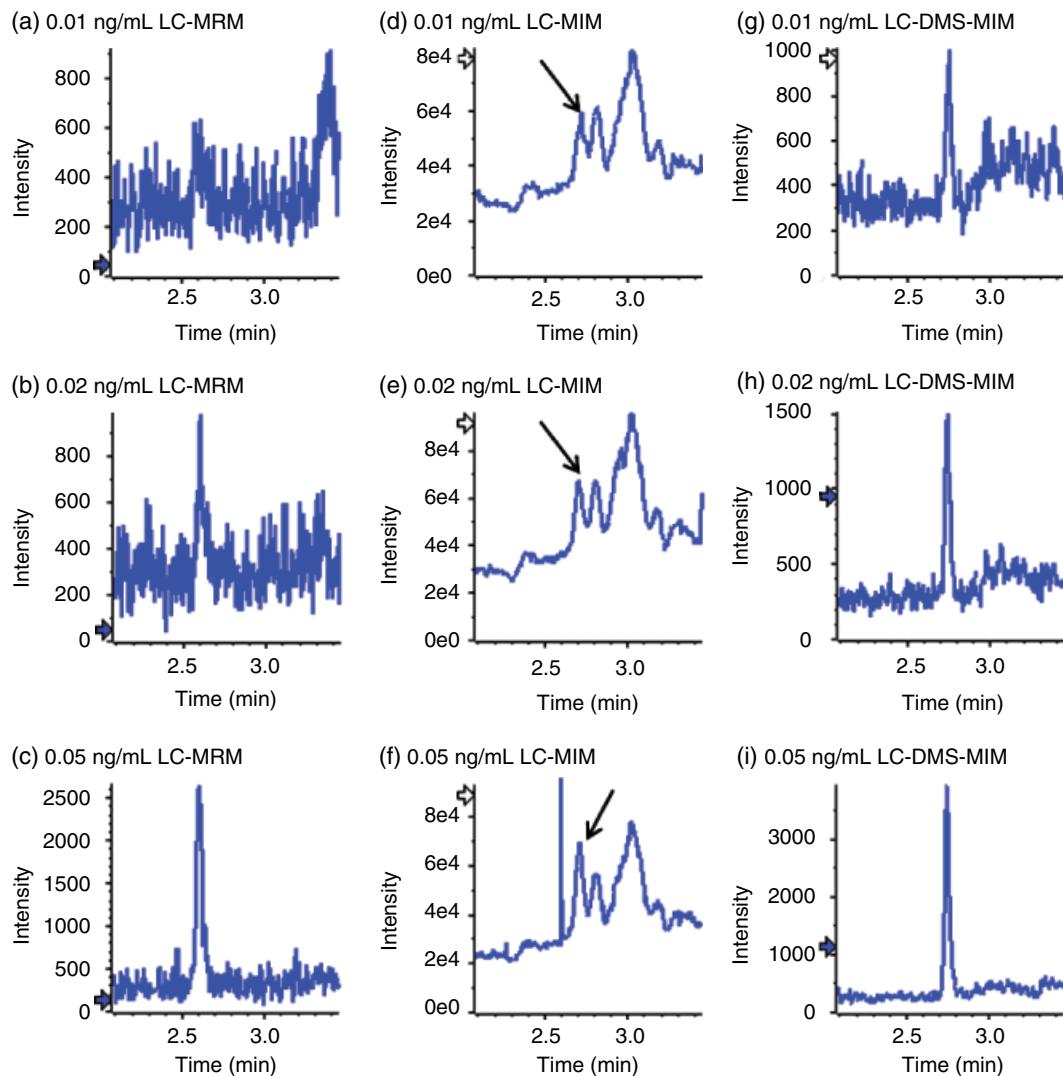
An LC-DMS-MIM method in quantitation of pasireotide, a therapeutic cyclic peptide, had been successfully developed and validated, in compliance with the regulated bioanalysis (Fu et al., 2016). While this technology was applied for a drug candidate, its principle can be easily adopted for endogenous biomarker analysis. In positive ESI, the protonated molecule of pasireotide exists in the doubly charged ion (Figure 11.2a). However, the doubly charged precursor ion of pasireotide showed inefficiency CID fragmentation. At collision energy (CE) of 23 eV, a doubly charged product ion was observed at  $m/z$  427.4 (Figure 11.2b) with a signal intensity of about 20-fold lower than the intensity of the precursor ion in Q1 scan. Further increase in CE to 30 eV led to production of several low abundance and small fragment ions in the MS/MS spectrum (Figure 11.2c). The optimized MS/MS conditions with CE of 23 eV were incorporated into the LC-MRM method with MS/MS transition of  $m/z$  524.4 →  $m/z$  427.4. However, the intended LC-MRM LLOQ of 0.01 ng/mL was unachievable (Figure 11.3a), neither at the 0.02 ng/mL level (Figure 11.3b). The reachable LLOQ was 0.05 ng/mL (Figure 11.3c). Although LC-MIM of  $m/z$  524.4 →  $m/z$  524.4 achieved good signal intensity when injecting the neat solutions, a high baseline due to matrix background was observed when injecting plasma samples, hindering the effort to achieve the desirable sensitivity of 0.01 ng/mL. Adjustments were made to decrease the baseline by increasing the collision energy while maintaining reasonable LC-MS signals in MIM. At CE of 15 eV, the LC-MS baseline was



**Figure 11.2** Representative mass spectra of pasireotide obtained from QTRAP 6500 system Q1 (a) scan (the doubly charged molecular ion was observed at  $m/z$  524.4) and Q3 scan of the doubly charged molecular ion of  $m/z$  524.4 under collision energy of 23 eV (b) and 30 eV (c). Source: Fu et al. (2016). Reproduced with permission of American Chemical Society.

decreased as compared with that observed at CE of 5 eV, but it was still high up to  $2 \times 10^4$  counts (Figure 11.3d). The pasireotide signal was concealed inside the matrix baseline. Adjacent matrix interference peak was also observed (Figure 11.3d–f). Even at 0.05 ng/mL level, the analyte peak shape was far from being acceptable for any possible integration. By combining LC-MIM with DMS, the intended LLOQ of 0.01 ng/mL was achieved with LC-DMS-MIM method with S/N ratio approximately 5 (Figure 11.3g). Among the three approaches, only the LC-DMS-MIM method yielded a quantifiable analyte peak, and the LC-MS/MS response was linear from 0.01 to 0.02 (Figure 11.3h) and to 0.05 ng/mL levels (Figure 11.3i). This clearly demonstrated the superiority

of LC-DMS-MIM over LC-MRM or LC-MIM alone for quantitative analysis of pasireotide in human plasma. The LC-DMS-MIM method was successfully validated in accordance with the health authority guidance and internal SOPs for regulated LC-MS bioanalysis with excellent accuracy and precision. The overall matrix factors were determined as 0.963, 0.899, and 0.975, respectively, for pasireotide at the low, mid and high concentration levels, similar to that measured using the LC-MRM approach. Apparently, LC-DMS-MIM does not alter the matrix effect observed using LC-MRM method. It is generally believed that matrix effect takes place in the ESI source region during ionization process. Since DMS device locates between the ESI source and



**Figure 11.3** A comparison of assay sensitivity by injecting the same sets of pasireotide human plasma extracts onto the LC-QTRAP 6500 system: Left panel, LC-MRM without differential ion mobility spectrometry (DMS): (a) 0.01 ng/mL, (b) 0.02 ng/mL, (c) 0.05 ng/mL. Middle panel, LC-MIM without DMS with optimized CE of 15 eV: (d) 0.01 ng/mL, (e) 0.02 ng/mL, (f) 0.05 ng/mL. Right panel, LC-DMS-MIM: (g) 0.01 ng/mL, (h) 0.02 ng/mL, (i) 0.05 ng/mL. Source: Fu et al. (2016). Reproduced with permission of American Chemical Society.

MS orifice, the ions entering the DMS cell already experience matrix effect, if there is any. By using pasireotide as a model compound, the unique advantages of DMS in combination with MIM over conventional MRM or MIM alone in quantitative LC-MS/MS bioanalysis of molecules with low CID efficiency are demonstrated. Most abundant precursor ions in Q1 and Q3 of the triple quadrupole MS instrument were selected by using MIM to ensure the high signal intensity, and DMS was used to minimize the high matrix baseline for achieving the desired assay specificity and sensitivity. For scientists using triple quadrupole mass spectrometry-based MRM for biomarker quantitation, the introduction of the

novel DMS-pseudo-MRM or DMS-MIM methodology can expand application of triple quadrupole instruments to quantify analytes that are not suited for MRM due to inefficiency CID fragmentation.

### 11.3 High-Resolution Mass Spectrometry

The advantages of LC-MRM bioanalytical methods used for the analysis of the post-dose samples are well known and include (a) excellent selectivity vis-à-vis biological endogenous components, metabolites, and formulation

ingredients; (b) good sensitivity with or without extensive cleanup of the biological samples; (c) acceptable assay precision and accuracy; (d) wide dynamic ranges, routinely achieving 4-order linear regressions; (e) short analysis turnaround time due to rapid chromatography and MRM acquisition speed; (f) straightforward post-acquisition data processing and quantitation; and (g) excellent long-term reproducibility and ruggedness, with easy method transferability between laboratories. The awareness among bioanalytical scientists of the pitfalls of LC-MRM bioanalytical methods associated with potential metabolite interference and matrix effect, such as ionization suppression/enhancement, is now high, and thus appropriate precautions are taken during method development and application. Consequently, appropriately enough, the LC-MRM technique is well entrenched in bioanalytical laboratories, which are equipped almost exclusively with triple quadrupole mass spectrometers. However, with the recent introduction of reasonably priced high-resolution accurate mass spectrometers with enhanced mass resolving power, mass accuracy, acquisition speed, and dynamic range, there is a growing interest in the bioanalytical community to explore the utility of liquid chromatography coupled with high-resolution accurate mass spectrometry (LC-HRMS) for the quantitation of drugs/drug candidates, metabolites, and biomarkers in post-dose samples. Recent publications have demonstrated proof of principle for the quantitation of small molecule drugs/drug candidates in plasma samples (Fung et al., 2011; Ramanathan et al., 2011; Xia et al., 2011).

The most distinct feature of LC-MRM is that only those precursor ions and their selected product ions, as specified by the MRM transitions used, are detected and thus no information about the other ions in the injected sample is acquired and recorded. This feature possesses the desirable attributes of enhanced selectivity and sensitivity arising from increased duty cycle, which is important for the accurate and precise quantitation of the target analyte. One drawback of this type of detection is the absence of information in regard to other compounds for post-acquisition analysis to answer questions that may arise related to the presence/absence of metabolites or the co-elution of other components that may cause unexpectedly low or high response of the target analyte. In comparison, the most distinct feature of LC-HRMS is its ability to detect the ions not only generated from the target analyte but also ions generated from other compounds in the same injected sample. Accordingly, post-acquisition data queries can be conducted if the need arises to obtain information about additional components other than the target analyte. Thus, qualitative information about metabolites, biomarkers, phospholipids, and formulation agents can be obtained from the

same sample injection used for the quantitation of the target analyte. This feature of LC-HRMS offers a significant potential advantage over LC-MRM. Previous reports (Fung et al., 2011; Ramanathan et al., 2011) showed that LC-MRM methods are, for most compounds, more sensitive than LC-HRMS methods. However, TOF-based LC-HRMS methods have achieved LLOQ of 1 and 5 ng/mL for at least 50–90%, respectively, of model compounds in plasma, following a simple sample extraction using plasma protein precipitation procedure.

It is important to evaluate the mass resolving power required for LC-HRMS bioanalytical methods to provide the selectivity needed for bioanalysis. It should be noted that accuracy of mass assignment, which is crucial in LC-HRMS, is affected both by the drift of the calibrated mass and by the resolving power, which is dependent on the complexity of the matrix. Recent publications (Kellmann et al., 2009; van der Heeft et al., 2009; Kaufmann et al., 2010) examined the correlation of mass resolving power and selectivity for residue analysis in matrices such as tissue, honey, food, and animal feed; however, plasma or serum, which is the most common matrix used in biomarker studies, was not included. Another important aspect in LC-HRMS method development is the effect of the mass extraction window (MEW) used in the post-acquisition data processing to obtain the extracted ion chromatogram (XIC) for the target analyte from the total ion chromatogram (TIC). The accuracy of the area value obtained for the target analyte chromatographic peak in the XIC is dependent on using the optimum MEW. Thus, understanding this step of method development and selecting the optimum MEW, which is related to the resolving power used during the TOFMS acquisition, is crucial in developing a sound and rugged LC-HRMS bioanalytical method.

Xia reported a systematic evaluation for assessing the overall selectivity that can be attained with LC-HRMS to differentiate analytes from potential interferences from endogenous components in plasma, the most common biological matrix used in quantitative bioanalysis (Xia et al., 2011). This is demonstrated on a TOF-based system by assessing selectivity as a function of resolving power and MEW using 153 small molecule analytes.

Mass resolving power is the mass spectrometer's ability to resolve two adjacent mass peaks. MEW is a parameter used for obtaining the XIC of the analyte of interest from a TIC. Mass resolving power, defined in terms of FWHM, is as follows:

$$R = \frac{M}{\text{FWHM}} \quad (11.1)$$

where  $R$  is the resolving power,  $M$  is the  $m/z$  of the compound of interest (when  $z=1$ ,  $M$  equals the mass of the compound), and FWHM is the full width at half

maximum. Thus, FWHM can theoretically be determined for a given *m/z* and resolving power as follows:

$$\text{FWHM} = \frac{M}{R}$$

For near-baseline separation of two adjacent masses, the difference between the two masses should be at least twice the FWHM. As shown in Table 11.1, for the first pair consisting of *m/z* 609.2198 (M1) and *m/z* 609.3416 (M2), the difference between the two masses is 0.1218 Da or 200 ppm. At 10 k resolving power, the calculated FWHM is 0.0609 Da or 100 ppm, and the corresponding values at 20 k resolving power are 0.0305 Da or 50 ppm. Thus, the difference between M1 and M2 is  $\geq 2$  times FWHM at either 10 or 20 k resolving power, indicating near-baseline resolution between the two masses at either resolving power. For the second pair of masses of *m/z* 609.2503 (M1a) and *m/z* 609.3112 (M2a), the difference between the two masses is 0.0609 Da or 100 ppm. Thus, the difference between M1a and M2a is  $\geq 2$  times FWHM only at 20 k resolving power, indicating near-baseline resolution between the two masses only at 20 k resolving power.

When obtaining XIC from a TIC, the MEW should be at least  $\pm$ FWHM if the intention is to cover the entire analyte mass peak width. Since there is no obvious advantage in using MEW larger than the entire mass peak width, we designated the maximum MEW to be equal to  $\pm$ FWHM. Maximum MEW for *m/z* 609.2807 ranged from  $\pm 0.00305$  to  $\pm 0.0609$  Da for resolving power of 20–10 k. The corresponding MEW values expressed in terms of ppm range from  $\pm 5$  to  $\pm 100$  ppm. It is evident that, at a given resolving power, the maximum MEW value expressed in  $\pm$ Da increases in direct relation to the mass value. In contrast, the maximum MEW value expressed in  $\pm$ ppm at a given resolving power remains the same irrespective of the mass value. Accordingly, the maximum MEW at 10 and 20 k resolving power are  $\pm 100$  and  $\pm 50$  ppm, respectively, no matter what the mass value is.

In order to evaluate the overall selectivity of the HRMS, the detection of potential endogenous interferences in plasma was assessed by injecting acetonitrile-precipitated blank human plasma extract into the LC-HRMS system using 10 and 20 k resolving power (Xia et al., 2011). From the resultant TIC of a 3.5 min gradient run, they assessed the detection of human plasma endogenous components by generating XICs of the exact masses of protonated precursor ions of 153 analytes using different MEWs. The MEWs of 5, 10, and 20 ppm were used in 10 and 20 k resolving power. A plasma endogenous component peak was reported as present if the height of the chromatographic peak in the XIC was  $\geq 2000$  counts and the peak area was  $\geq 3000$  counts., which is based on

assessing the minimum peak response that is required to achieve an acceptable LLOQ.

By examining the number of plasma components observed for each model compound across the four MEW values for each resolving power, the number of the components detected generally increase with decrease in resolving power or increase in MEW. A clear difference is seen in the total number of observed endogenous peaks between the 10 and 20 k resolving power at a given MEW or between the different MEWs at a given resolving power. It is apparent that the number of the compounds that show endogenous peaks is a function of both the resolving power and MEW.

To better explain the difference in the observed selectivity, Figure 11.4 shows the XIC (*m/z* 559.2603) of Lipitor in the profile mode at 10 and 20 k resolving power with MEWs of 5, 20, and 50 ppm. At the retention time of 2.26 min, a plasma endogenous component is detected at *m/z* 559.3011 under 10 and 20 k resolving power, which represents a difference of +40.8 mDa (73 ppm) and +38.6 mDa (69 ppm), respectively, from the Lipitor mass. The calculated FWHM for the endogenous component mass is 55.9 mDa (100 ppm) and 28.0 mDa (50 ppm) at 10 and 20 k resolving power, respectively. As illustrated in Figure 11.4A1, when applying MEW of 5 ppm at 10 k resolving power, the theoretical Lipitor mass (*m/z* 559.2603) is at the shoulder of the plasma endogenous mass, which leads to the detection of a plasma endogenous peak at the retention time of 2.26 min (Figure 11.4B1). However, when applying the same MEW of 5 ppm at 20 k resolving power, the Lipitor mass is better resolved from the plasma endogenous mass, and hence no plasma endogenous peak is detected at the corresponding retention time. As the MEW is increased to 20 ppm, the inclusion of the endogenous mass into the Lipitor mass increases (Figure 11.4A2), which results in the detection of the endogenous peak at 10 or 20 k resolving power (Figure 11.4B2). The same trend continues as the MEW is increased to 50 ppm (Figure 11.4A3 and B3). The best specificity was achieved at the higher resolving power of 20 k and narrowest MEW of 5 ppm.

It is clear from the aforementioned data that the 20 k resolving power is significantly better than the 10 k resolving power in the selectivity achieved for the analytes tested in human plasma acetonitrile extract. Considering that this type of plasma extract is normally considered to be a crude extract when compared with an extract obtained from liquid–liquid extraction (LLE) or solid–phase extraction (SPE), the finding that only approximately 10% of the 153 model compounds tested showed at least one endogenous component at 20 k resolving power and 5 ppm MEW demonstrates that excellent selectivity can be achieved for bioanalytical methods under such resolving power and MEW. Hence,

**Table 11.1** Calculation of resolving power required to resolve two adjacent mass peaks: demonstrated for resolving M1 from M2 and M1a from M2a.

Mass 1	Mass 2	10 k resolving power			20 k resolving power			Mass 2 minus mass 1	Resolution between M1 and M2 and between M1a and M2a		
		Difference between two masses required for near-baseline separation at 10 k resolving power			Difference between two masses required for near-baseline separation at 20 k resolving power						
		FWHM at 10 k resolving power	(Da) <sup>a</sup>	(ppm) <sup>b</sup>	FWHM at 20 k resolving power	(Da) <sup>d</sup>	(ppm) <sup>e</sup>				
(Da)	(Da)	(Da) <sup>a</sup>	(ppm) <sup>b</sup>	(ppm) <sup>c</sup>	(Da) <sup>d</sup>	(ppm) <sup>e</sup>	(ppm) <sup>f</sup>	(Da or ppm)			
609.2198 (M1)	609.3416 (M2)	0.0609	100	200	0.0305	50	100	0.1218 (Da) 200 (ppm)	M1 and M2 are near baseline resolvable at both 10 k and 20 k resolving power, since their difference is 200 ppm		
609.2503 (M1a)	609.3112 (M2a)	0.0609	100	200	0.0305	50	100	0.0609 (Da) 100 (ppm)	M1a and M2a are near baseline resolvable only at 20 k resolving power, since their difference is only 100 ppm		

Source: Xia et al. (2011). Reproduced with permission of John Wiley & Sons, Ltd.

<sup>a</sup> It is calculated as 609.2198/10,000 for M1 and 609.2503/10,000 for M1a at 10 k resolution.

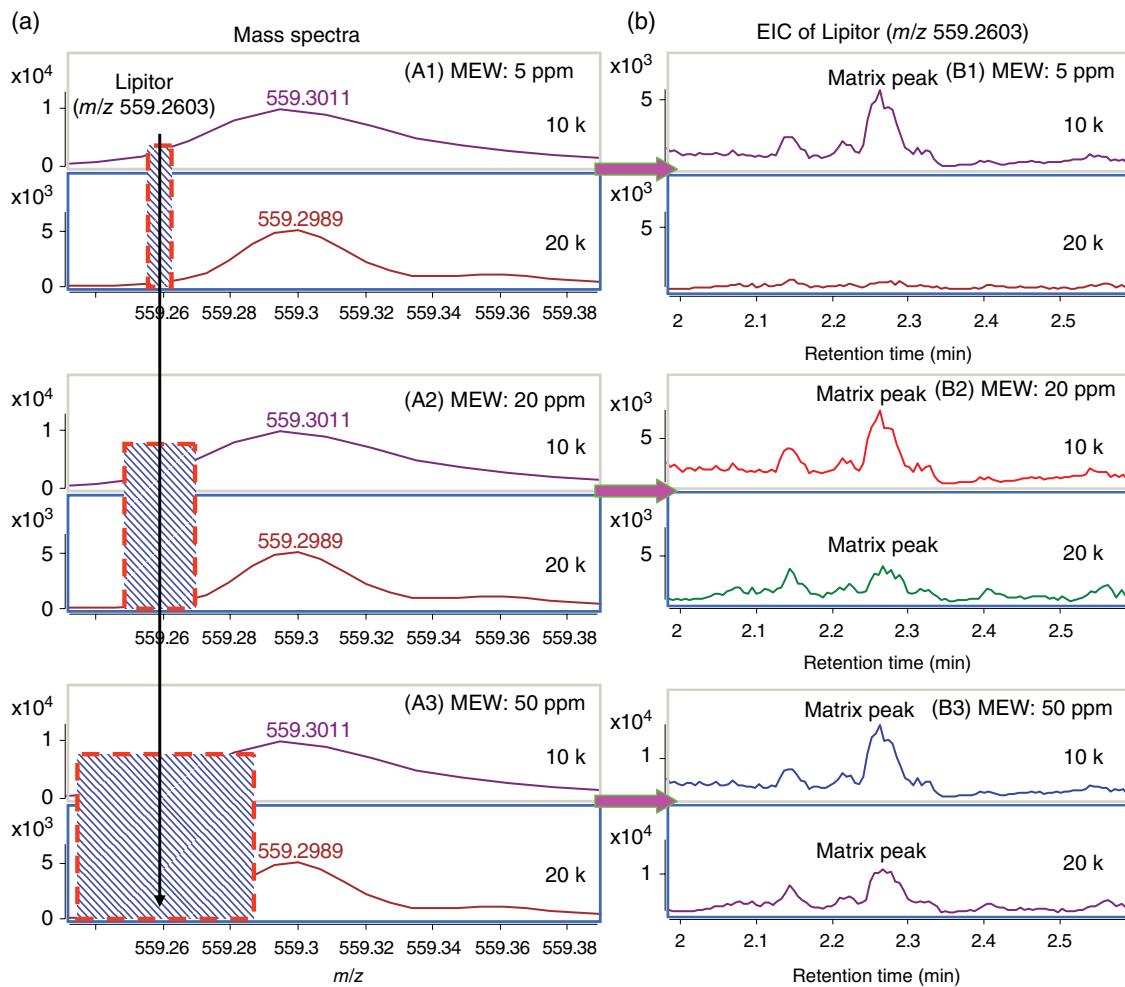
<sup>b</sup> It is calculated as  $(\text{FWHM}/\text{mass}) \times 10^6$ , where both FWHM and mass are expressed in Da. Alternatively, it can be calculated as  $(1/R) \times 10^6$ , where R is resolving power ( $R = 10,000$  in this case), since FWHM/mass can be shown to be equal to  $1/R$ .

<sup>c</sup> The difference between two neighboring masses is expressed as ppm. It is calculated as  $2 \times \text{FWHM}$  ppm at 10 k resolving power.

<sup>d</sup> It is calculated as 609.2198/20,000 for M1 and 609.2503/20,000 for M1a at 20 k resolving power.

<sup>e</sup> It is calculated as  $(\text{FWHM}/\text{mass}) \times 10^6$ , where both FWHM and mass are expressed in Da. Alternatively, it can be calculated by  $(1/R) \times 10^6$ , where R is resolving power ( $R = 20,000$  in this case), since FWHM/mass can be shown to be equal to  $1/R$ .

<sup>f</sup> The difference between two neighboring masses is expressed as ppm. It is calculated as  $2 \times \text{FWHM}$  ppm at 20 k resolving power.



**Figure 11.4** Illustration of the effect of resolving power and extraction window in the profile mode using the Lipitor ( $m/z$  559.2603) XIC obtained from the TIC of the acetonitrile-precipitated blank human plasma extract with three different MEWs and 10 k and 20 k resolving power, with the corresponding mass spectra at the retention time of 2.26 min, where the XIC exhibits endogenous component. Left panel (A): mass spectra at 2.26 min using MEW of: (A1) 5 ppm; (A2) 20 ppm; (A3) 50 ppm. Right panel (B): XIC of Lipitor obtained using MEW of: (B1) 5 ppm; (B2) 20 ppm; (B3) 50 ppm. Source: Xia et al. (2011). Reproduced with permission of John Wiley & Sons, Ltd.

20 k resolving power can be considered adequate for the accurate determination of analytes in plasma.

With the acquisition resolving power of 20 k fixed, the next question is, what MEW should be used in practice? The results show that the selectivity for 20 k resolving power is the highest at MEW of 5 ppm, which is only one-tenth of the maximum MEW (or FWHM). Thus, the optimum MEW is ideally a small fraction of the FWHM and thus inversely related to the resolving power used. A component that co-elutes with the analyte peak but is mass resolved during the acquisition would be co-integrated with the analyte chromatographic peak if the MEW used is wide enough to include the mass of the co-eluting component. On the other hand, using MEWs narrower than the optimum value may not improve selectivity since endogenous components whose masses fall within

such a narrow window may not be mass resolved from the target analyte. When using a very narrow MEW, some or all of the data points in the analyte peak could be missed due to inaccuracy in analyte mass assignment, which could be caused by the presence of a co-eluting component not mass resolved from the analyte or by the drift of calibrated mass. Such occurrences would result in unacceptable accuracy and precision of a bioanalytical method due to inadequate number of data points, or, worse, in false negative (absence of the analyte chromatographic peak). Therefore, a resolving power of 20 k and optimum MEW are generally adequate to achieve accurate and precise quantitation in plasma matrix.

Along with HRMS full-scan method, HR-MS/MS method is an additional method of choice in quantitation of molecules in biological matrices. Because of the high

resolution and mass accuracy in MS/MS spectra, the specificity is even better than HRMS in analysis of molecules from the complex biological matrices. Typical HR-MS/MS method consisted of HRMS full scan and target MS/MS scan to collect both precursor ions and fragment ions of the target molecules. Quantitative bioanalysis is accomplished by obtaining XIC using a specific HR-MS/MS fragment ion or summing up multiple fragment ions. Qualitative analysis is done by searching HRMS full-scan spectra to confirm the presence or absence of other interesting components. This approach enables scientists to do both qualitative and quantitative analysis simultaneously via a single injection. However, this target TOF-MS/MS approach does not provide useful MS/MS fragmentation for the unknown molecules due to the fact that it predefines the target precursors for HR-MS/MS acquisition. Although information-dependent acquisition (IDA) can generate both HRMS and HR-MS/MS information, it is sometimes unable to obtain HR-MS/MS for trace level of components especially from complex biological matrices. Recently, a data-independent acquisition via sequential window acquisition of all theoretical (SWATH from Sciex) was introduced to acquire an unbiased record of all fragments of all precursors within one SWATH acquisition isolation window. These data acquisition platforms generate fragments for all eluting components along with their precursor ions in a single LC-MS run. For example, the Q1 mass range interrogated was  $m/z$  100–1000 with Q1 transmission window set at 30Da for a total of 32 segments to cover the whole mass region of 1000Da. This provides high quality HR-MS/MS data for not only structural assignments of unknown components but also quantitative bioanalysis.

Armed with the knowledge discussed earlier, LC-HRMS was successfully used to analyze endogenous biomarkers. For example, a detailed study on characterization and quantification of oxyntomodulin (OXM) in human and rat plasma using LC-HRMS was reported by the Lilly researchers (Cox et al., 2016). OXM is a peptide consisting of 37 amino acids and is a dual agonist of the glucagon-like peptide-1 (GLP-1) and glucagon receptors. It has garnered interest as a potential therapeutic agent for obesity and type 2 diabetes and has been the focus of extensive preclinical and clinical research

because of its physiological effects on weight loss, food intake, and energy balance. In the reported study, immunoaffinity capture coupled with LC-HRMS was used to quantify OXM and its primary catabolites. OXM 1–37 was detected along with its three N-terminal catabolites generated by dipeptidyl peptidase 4 (DPP-4)-mediated hydrolysis. The assay was directly applied to measure OXM and its N-terminal catabolites in both rat and human plasma. The data obtained allow for a kinetic comparison of the rates of catabolism between species and add to the growing base of knowledge for OXM (Cox et al., 2016).

In another publication, validation of a dual LC-HRMS platform for clinical metabolic diagnosis in serum, bridging quantitative analysis, and untargeted metabolomics was reported (Gertsman et al., 2014). Using LC-HRMS with a Sciex TripleTOF mass spectrometer with two different types of pentafluorophenyl (PFP) stationary phases, employing both positive and negative ionization, a hybrid quantification and discovery platform was developed and validated. This dual-PFP LC-MS platform quantifies over 50 clinically relevant metabolites in serum while simultaneously collecting high-resolution and high mass accuracy full scans to monitor all other co-eluting nontargeted analytes.

## 11.4 Conclusions

There are many challenging issues in LC-MS bioanalysis of biomarkers, and those challenges are different for every molecule. The technologies and concepts covered in this chapter can be implemented to resolve some challenging selectivity and sensitivity issues in quantitative LC-MS bioanalysis. DMS can be considered as a method of choice for reducing high chemical background noise, eliminating matrix interferences, and separating isobaric metabolites in quantitative LC-MS bioanalysis. HRMS full scan and HR-MS/MS or SWATH acquisition are alternative methodologies for not only quantitative bioanalysis but also qualitative analysis of unknown components via a single LC-MS run. Nevertheless, it should be emphasized that well designed strategies are essential in dealing with the issues of assay selectively and sensitivity in LC-MS bioanalysis.

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## 12

### Biomarker Quantitation Using Relative Approaches

*Shane M. Lamos and Katrina E. Wiesner*

*Department of Chemistry, Saint Michael's College, Colchester, VT, USA*

#### 12.1 Introduction

A complete understanding of biological system regulation, namely, the -omics sciences such as genomics, proteomics, and metabolomics, can only be realized when all of the important regulatory molecules, “biomarkers,” can be systematically identified and quantified. Much research effort has been devoted to developing new analytical platforms for meeting these modern -omics challenges.

Mass spectrometry (MS) provides the necessary analytical precision over the wide range of biomarker molecular weights to make it the bedrock of modern -omics approaches. Significant challenges remain, however, due to the widely varied chemical structures of genes, proteins, and metabolites.

With the advent of modern mass spectrometers with high mass accuracy, precise quantitation of biomolecules using small mass differences created by the incorporation of stable isotopes has become a reliable paradigm for developing new quantitative methodologies. These isotopic labeling approaches can be further delineated by the type of quantitative measurements they give, absolute versus relative, and the relative approaches can be further defined by their mode of isotope incorporation, enzymatic, metabolic, chemical mixed isotope, and chemical isobaric. The use of spiked standards, known as stable isotope dilution (SID), has long been the “gold standard” for the absolute quantitation of biomarkers. SID has been used extensively in proteomic, lipidomic, and metabolomic studies (MacDonald et al., 2012; Snyder et al., 2014). A simple spiking experiment using the isotopic biomarker allows for the accurate quantitation of the biomarker of interest. SID, while a powerful approach, is limited in number and in scope for a number of reasons including high manufacturing costs

associated with the isotopic biomarker, limited availability of isotopic biomarkers, and the lack of information discovered about -omics members for which the study did not include an SID isotopic form (Chapman et al., 2012).

In contrast to absolute quantitation, relative quantitation performed with the various labeling approaches (enzymatic, metabolic, mixed isotope, and isobaric) attempts to elucidate the percent or portion of a biomarker present in a sample relative to the amount of that biomarker found in one or more other related biological samples, thus providing critical information about biomarker expression levels between samples. Relative quantitation represents robust methodologies for cost- and time-efficient approaches for understanding the important roles that biomarkers play in cellular and organismal regulation (Table 12.1).

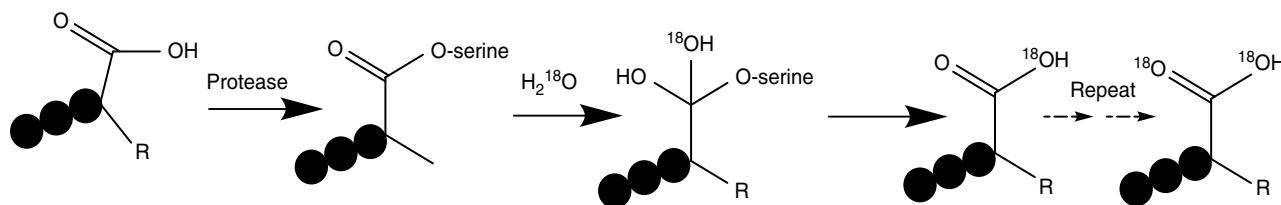
#### 12.2 Relative Quantitation Isotope Labeling Approaches

##### 12.2.1 Enzymatic Isotopic Incorporation

Enzymatic isotopic labeling is the lowest cost method among the four important relative labeling approaches. The most common form of enzymatic labeling involves the incorporation of  $^{18}\text{O}$  atoms at the C-terminus of proteolytic fragments wherein the two native  $^{16}\text{O}$  are both replaced by  $^{18}\text{O}$  atoms by enzymatic exchange in  $\text{H}_2^{18}\text{O}$ . This proteolytic labeling method can be used to make relative (sample 1, light; sample 2, heavy) quantitative measures of protein expression levels after light and heavy labeled samples are pooled and analyzed by LC-MS. All peptides contain a carboxyl group, and so this type of enzymatic labeling also represents a global

**Table 12.1** Isotope labeling approaches for relative quantitation of biomarkers.

Enzymatic isotopic incorporation	Description	Benefits	Limitations
<sup>18</sup> O labeling	Incorporation of <sup>18</sup> O at C-terminus proteolytic fragments	Low cost, carries PTMs, global protein quant., fast, no post-labeling cleanup, applicable to samples at low femtomole level	Reversibility of enzymatic incorporation, incomplete enzymatic incorporation
TOSIL	Tandem <sup>18</sup> O stable isotope labeling	Third <sup>18</sup> O incorporation, greater isotopic shift	
<sup>18</sup> O labeling with iTRAQ	Coupled with multiplexing reagent (iTRAQ)	Global quant. for glycosylated, non-glycosylated peptides	
Metabolic isotopic incorporation	Description	Benefits	Limitations
SILAC	Stable isotope labeling by amino acids in cell culture	Early isotopic labeling (minimal handling bias)  In vivo labeling is quantitative  Thousands of proteins labeled at minimal cost  Highest accuracy  Isotopically labeled proteins easily predicted  MaxQuant freely available for analysis  PTMs can be determined, kinetic workflows possible	Some cells harder to grow in dialyzed serum  Special diets necessary for isotopic incorporation  Cannot be used for humans in vivo  High performance mass spec. required to collect data
SILAC spike-in	SILAC standard added to sample for analysis	Human clinical studies	Not a global approach
Super-SILAC	Internal standards from cell mix, global quant.	Can study clinical samples, secretomes, PTMs, organelle proteomes with higher accuracy and precision	Requires multiple cell lines
Neutron-encoding SILAC	Utilizes technology that is highly sensitive to isotopes	Multiplexed proteomics	Requires highly sensitive MS (expensive)
pSILAC	Pulsed SILAC, concentrations measured over time	Kinetic data of protein expression and degradation	
Chemical labeling (nonisobaric)	Description	Benefits	Limitations
ICAT	Isotope-coded affinity tag	Identification of low abundance molecules	Limited sequencing info, precision lost for low abundance peptides (nonquantitative)
cICAT	Cleavable ICAT	Simplifies mass spec. analysis	
Reductive methylation	Amine-targeting label, adds methyl group	Low cost, fast rate, mild conditions, high specificity to amino groups, high labeling and ionization efficiency, stable post-derivative products, multiplexed analysis	Limited isotope incorporation
Chemical labeling (isobaric)	Description	Benefits	Limitations
iTRAQ	Isobaric tags for relative and absolute quantification	Commercialized, high sensitivity, better reproducibility, allows analysis of low abundance kinases, multiplex anal.	Less accurate
TMT	Tandem mass tags	Multiplex, efficient, consistent, flexible, software to analyze	Ratio distortions, expensive
Deuterium labeling (DiLeu)	N,N-dimethyl leucines	Four-plexed analysis, low cost	Chromatographic retention differences



**Figure 12.1**  $^{18}\text{O}_2$  labeling of peptide by serine protease.

approach as all peptides can be targeted and the only reagent remaining after labeling is water, thus removing any post-labeling cleanup steps.

While enzymatic labeling provides a low cost and expedited methodology for peptide quantitation, it is somewhat constrained in its utility due to the reversibility of the enzymatic incorporation of the  $^{18}\text{O}$  atoms and the sometimes reported incomplete nature of the labeling. The introduction of  $^{18}\text{O}$  is usually catalyzed by one of several serine proteases: trypsin, Glu-C protease, Lys-C protease, or (slow) chymotrypsin (Figure 12.1) (Fenselau and Yao, 2009). In the binding site of the protease, the amino acid residue to be labeled is covalently bound in a tetrahedral intermediate. A water ( $\text{H}_2^{18}\text{O}$ ) molecule acts as a nucleophile and attacks the tetrahedral intermediate, adding  $^{18}\text{O}$  to the carboxyl group of the residue. The C-terminal residue generated by this proteolytic process is still recognized by the protease, and the peptides are bound repeatedly using the same covalent mechanism. If the peptide products are incubated with the catalytic enzyme in  $\text{H}_2^{18}\text{O}$ , microreversibility will eventually allow the level of  $^{18}\text{O}$  in the peptides and the level of  $^{18}\text{O}$  in the solvent to equilibrate, resulting in incomplete labeling (Fenselau, 2007). Several researchers have developed new methods to limit the drawbacks of enzymatic  $^{18}\text{O}$  incorporation including enhancing C-terminus protonation and thus binding to the proteolytic enzyme by lowering the pH of the solution and using immobilized proteases that can be removed promptly after successful  $^{18}\text{O}$  incorporation to prevent back-reactions with  $^{16}\text{O}$  later (Lang et al., 2013; Castillo Mary et al., 2014).

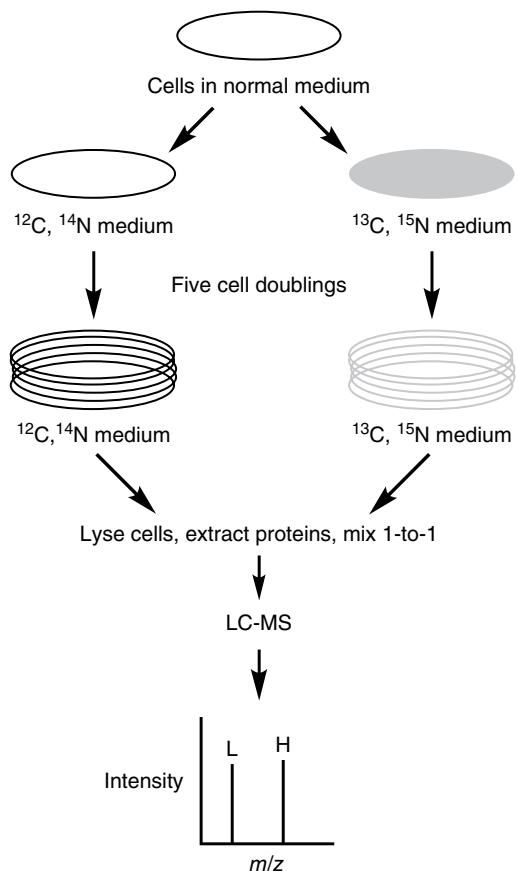
In addition to the application of enzymatic labeling with  $^{18}\text{O}$  noted above, enzymatic labeling has recently been coupled with other analytical identification and quantitation techniques to extend the scope of this approach. Sites of glycosylation have been determined by incubating C-terminus  $^{18}\text{O}$  enzymatically labeled peptides with *N*-glycopeptidase F in  $\text{H}_2^{18}\text{O}$ , causing a third  $^{18}\text{O}$  incorporation at the site of deglycosylation (Shakey et al., 2010; Kaji and Isobe, 2013). This new approach termed “tandem  $^{18}\text{O}$  stable isotope labeling” (TOSIL) has been used to successfully quantify 86 N-glycosylation sites in the serum of an ovarian cancer patient (Liu et al., 2010). In an effort to overcome the binary nature of

$^{18}\text{O}$  labeling studies ( $^{16}\text{O}$  versus  $^{18}\text{O}$ ), Lu and Liu have introduced another enzymatic coupled approach this time with the multiplexing reagent iTRAQ (Zhang et al., 2012). All peptides from four biological samples were labeled with unique iTRAQ reagents (114, 116, 115, and 117) before being either  $^{16}\text{O}$  (114 and 116 samples) or  $^{18}\text{O}$  (115 and 117 samples) enzymatically labeled with *N*-glycopeptidase F in  $\text{H}_2^{18}\text{O}$  as above. The four doubly labeled samples were pooled and analyzed in a multiplex fashion giving global quantitative information about both glycosylated and non-glycosylated peptides.

Enzymatic  $^{18}\text{O}$  labeling is a universal strategy that leads to labeled peptides from all kinds of proteins carrying any kind of posttranslational modification (PTM). It is a residue-specific catalytic method. It is applicable to samples at the low femtomole level. The reagents are cost effective and readily available. The light and heavy peptide pairs co-elute in high pressure liquid chromatography, and manual interpretation of MS/MS spectra is facilitated by the C-terminal  $^{18}\text{O}$  labels (Fenselau and Yao, 2009).

### 12.2.2 Metabolic Isotopic Incorporation

Metabolic isotopic labeling involves the incorporation of isotopes in living organisms as they grow in isotopically enriched media. Many strategies for isotopic incorporation can be envisioned, but most successful strategies developed in the past 20 years have focused on the incorporation of isotopes as found in essential feed of living cells. In practice, metabolic isotopic incorporation involves a simple workflow and analysis (Figure 12.2). Two populations of cells are grown in either a “light” (normal) feed or a “heavy” (isotopically enriched) feed. Complete labeling is usually achieved after five cell doublings (Mann, 2006). After labeling, the cells are mixed, proteins are extracted and proteolyzed, and finally the resulting peptides are analyzed by LC-MS/MS. Paired peaks are compared wherein relative ion count intensities relay quantitative information. The greatest advantage of metabolic isotopic incorporation versus others is the very early incorporation of the isotopes. This early incorporation of isotopes into labeled proteins leads to a minimization of bias due to later handling errors that are



**Figure 12.2** Metabolic isotopic incorporation workflow.

inherently harder to manage for processes that incorporate isotopes at a later stage in the analysis process.

Several isotopically enriched feed platforms have been developed to support metabolic isotope incorporation including global  $^{15}\text{N}$  labeling (Wu et al., 2004), and stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002). Ideally, SILAC amino acids should be essential for cell survival. Leucine, lysine, and methionine have been used as they are vital amino acids. Arginine is not an essential amino acid, but it has shown to be essential for many cultured cell lines and has worked quite well in SILAC labeling, despite its conversion to proline. Tyrosine is another nonessential amino acid that has been used for SILAC. It was used to identify the substrates of tyrosine kinase and to investigate the dynamics of tyrosine phosphorylation of proteins (Chen et al., 2015).

Commonly used isotopic atoms are deuterium,  $^{13}\text{C}$ , and  $^{15}\text{N}$ . In early SILAC studies,  $^2\text{H}$ -labeled leucine was used as the labeled amino acid, but a chromatographic shift during reversed-phase chromatography for the deuterium-labeled peptides compromised the accuracy of quantification.

The most commonly used isotopic amino acids added to the medium today are  $^{13}\text{C}$ - and  $^{15}\text{N}$ -labeled lysine and arginine. Trypsin cleavage at these isotopically labeled lysine and arginine residues ensures broad isotopic coverage of proteomic peptides. A disadvantage to using arginine is that it can be converted to proline during cell division, leading to potential inaccuracies and complicating the final quantitation. One strategy to limit this problem is to reduce the concentration of arginine in the media to a level where conversion to proline is unfavorable. This problem can be eliminated altogether by adding sufficient amounts of unlabeled proline to the media as well as ensuring that sufficient arginine is present to prevent back-conversion of proline to arginine (Elliott et al., 2009).

The SILAC approach to protein quantitation (Figure 12.2) has found its most robust use in expression-proteomics experiments. These expression-proteomics studies have given keen insights into protein expression changes that occur during cell differentiation. Some examples of SILAC-based expression proteomics include muscle cell differentiation (Cui et al., 2009), stem cell differentiation (Kratchmarova et al., 2005), and proteomic changes after biological or chemical stimulus (Gruhler et al., 2005; Xiong and Wang, 2010). SILAC is also ideally suited for studying protein expression in subcellular organelles, such as the nucleus (Hwang et al., 2006), or nucleolus (Hinsby et al., 2006).

Other interesting applications of SILAC include studies of the secretome. The secretome is represented by all proteins that have been secreted by a specific cell. Oftentimes these secreted proteins are important markers of disease such as various forms of cancer (Formolo et al., 2011). In one such case, Marimuthu et al. used SILAC to study the secretome of gastric cancer. Gastric cancer is a commonly occurring cancer in Asia and one of the leading causes of cancer deaths (Marimuthu et al., 2013). However, there is no reliable blood-based screening test for this cancer. Identifying proteins secreted from tumor cells would lead to the discovery of clinically useful biomarkers for early detection of gastric cancer. A SILAC-based quantitative proteomics approach was employed to identify secreted proteins that were differentially expressed between neoplastic and nonneoplastic gastric epithelial cells. Proteins from the secretome were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and strong cation exchange (SCX)-based fractionation, followed by mass spectrometric analysis on an LTQ Orbitrap Velos mass spectrometer. Immunohistochemical labeling was employed to validate a subset of candidates using tissue microarrays. Marimuthu et al. identified 2205 proteins in the gastric cancer secretome, of which 263 proteins were overexpressed greater than fourfold in gastric cancer-derived cell lines as compared with

nonneoplastic gastric epithelial cells. Three candidate proteins—proprotein convertase subtilisin/kexin type 9 (PCSK9), lectin mannose binding 2 (LMAN2), and PDGFA-associated protein 1 (PDAP1)—were validated by immunohistochemical labeling. This was the largest cancer secretome described to date in 2013. The novel biomarkers identified in the current study are excellent candidates for further testing as early detection biomarkers for gastric adenocarcinoma (Marimuthu et al., 2013).

SILAC has also found important utility in the study of PTMs including phosphorylation events. Early incorporation of isotope labels using SILAC allows PTMs to remain unmodified throughout later fractionation steps and incorporation at lysine and arginine sites guarantees that all tryptic peptides can be quantified, including those that have undergone PTMs. Both static and dynamic measurements of PTMs can be made as samples can be removed and quantified once or multiple times throughout an experimental time course.

Many PTMs act as regulatory switches for various signaling pathways and thus are key biomarkers for important cellular events. A major challenge of the post-genomics era is to define the connectivity of protein phosphorylation networks. Humphrey et al. (2013) quantitatively delineated the insulin signaling network in adipocytes by high-resolution MS-based proteomics. The data revealed the complexity of intracellular protein phosphorylation. The research team identified 37,248 phosphorylation sites on 5,705 proteins in this single cell type, with approximately 15% responding to insulin. They integrated these large-scale phosphoproteomics data using a machine learning approach to predict physiological substrates of several diverse insulin-regulated kinases. This led to the identification of an Akt substrate, SIN1, a core component of the mTORC2 complex. The phosphorylation of SIN1 by Akt was found to regulate mTORC2 activity in response to growth factors, revealing topological insights into the Akt/mTOR signaling network. The dynamic phosphoproteome described contained numerous phosphorylation sites on proteins involved in diverse molecular functions and should serve as a useful functional resource for cell biologists.

Numerous other SILAC-based approaches have been developed including SILAC spike-in for human clinical studies (Yu et al., 2009), super-SILAC for higher accuracy and precision (Geiger et al., 2010; Boersema et al., 2013), neutron-encoding SILAC for multiplexed proteomics (Merrill et al., 2014), and pulsed SILAC (pSILAC) for kinetic measurements of protein expression and degradation measured over a time course (Schwanhaeusser et al., 2009). A number of excellent reviews focused on SILAC-based approaches have recently been written (Ong, 2012; Dittmar and Selbach, 2015). In summary,

there are several important advantages to using a SILAC-based approach:

- Isotopic labeling occurs early in the workflow minimizing handling bias.
- In vivo labeling is quantitative after five cell doublings.
- Tens of thousands of isotopically labeled proteins can be made at minimal cost.
- Protein quantitation accuracy is among the best of all the relative isotopic labeling approaches.
- Isotopically labeled proteins can be easily predicted from the protein sequence.
- MaxQuant was specifically designed for SILAC data analysis and is freely available.
- PTMs can be determined and kinetic workflows are possible.

However, there are also several disadvantages:

- Some cells are harder to grow in the dialyzed serum required for SILAC.
- Special diets necessary for isotopic incorporation preclude its use with human beings.
- Arginine catabolism leads to heavy isotope incorporation into proline residues.

### 12.2.3 Chemical Labeling (Nonisobaric)

Chemical labeling strategies are complementary to the enzymatic and metabolic labeling strategies mentioned previously and are considerably more diverse in their design as a chemical labeling “toolbox” can be created to allow for efficient tagging of all chemical functionalities found in biomarkers. Proteins, for example, contain numerous functional groups that can be chemically labeled including sulphydryl groups, carboxyl groups, and amino groups. The functional groups available for metabolite tagging are even more diverse. The chemical tagging reagents themselves have incredible diversity due to the wide variety of stable isotopes that can be incorporated chemically and to the extent the researcher wishes to optimize the chemical label for improvements in chromatographic retention/separation and for spectral resolution/ionization efficiency. This extreme flexibility in design has allowed for unique chemical labeling approaches for performing analyses of many different kinds of samples on widely varied chromatographic and MS platforms.

Most nonisobaric chemical labeling strategies follow a similar workflow starting with the development of chemical tags (“heavy and light”), which are used to tag biomarkers containing a particular functional group. The resulting heavy- and light-tagged samples are mixed and fractionated before being analyzed by MS or MS/MS. Distinct biomarkers tagged with the heavy or light

chemical label fractionate together are analyzed by MS or MS/MS simultaneously giving a ratio of the two mass tag variants. This ratio yields the relative concentration of each biomarker between the two samples (Nakamura and Oda, 2007). There are a number of advantages to performing a chemical labeling strategy in this manner. First, the precision of relative quantification is improved by negating errors associated with multiple MS run analyses, such as ionization suppression or retention time differences between runs (Annesley, 2003; Smith et al., 2006). An equally important benefit of chemical labeling is gained through learning functional group identities—the one targeted by the reagent. Finally, well-designed reagents can improve chromatographic resolution, enhance ionization efficiency, provide clarifying MS/MS fragments (Bush et al., 2012), and can provide workflow time efficiencies by the creation of multiplexed isotopic reagents (Johnson, 2005; Smith and Rowland, 2008; Torde et al., 2013).

Isotope-coded affinity tags (ICATs) were the first commercially introduced chemical isotope labeling reagents (Gygi et al., 1999). The original ICAT reagent was a non-isobaric tag that consisted of three major components: an iodoacetamide group for forming covalent bonds with the sulphydryl group of cysteine side chain, a biotin affinity tag for isolating ICAT-tagged peptides, and an isotopically coded linker for relative quantification by MS. The biotin affinity tag, used to purify labeled peptides on a streptavidin column, greatly reduces sample complexity by fractionating those peptides that were tagged by the ICAT reagent. Unfortunately, some analytical precision is lost for low abundance peptides as binding and release of these peptides from streptavidin columns has been shown to be nonquantitative (Nesvizhskii et al., 2007). The limited abundance of the amino acid cysteine has meant that many peptide fragments, those that do not contain a cysteine, are not labeled or quantified using the ICAT reagent. In spite of these challenges, many significant biomarker studies have been successfully performed using the ICAT reagent (Petriz and Franco, 2014).

The original ICAT reagent was further improved upon by the inclusion of an acid-cleavable linker that greatly simplifies the mass spectroscopic analysis of labeled peptides. The so-called cleavable ICAT, cICAT, has likewise found widespread use in numerous proteomic studies (Filen et al., 2009; Costain et al., 2010). Zhou et al. used the cICAT to reveal 240 new secretory proteins while studying the secretome of adipocytes regulated by insulin (Zhou et al., 2009).

While the sulphydryl group of cysteines is relatively rare in peptide fragments, there is a permanent amino group on the N-terminus of every peptide and within the structure of every Lysine residue. The ubiquitous nature of the amino group has allowed for global peptide

quantitation using several amine-targeting labels (Shortreed et al., 2006; Boersema et al., 2009) including a reductive dimethylation labeling protocol. Reductive dimethylation was first introduced as a quantitative proteomic technique in 2003 (Hsu et al., 2003). Dimethylation is achieved through the reaction of an amine with formaldehyde, the methyl unit, in the presence of a chemical reducing agent, sodium cyanoborohydride. Reductive methylation has many important advantages including mild reaction conditions, low cost, fast reaction rates, high labeling and ionization efficiency, stable products, and the ability to do multiplexed analyses with the various isotopic formaldehydes and reducing agents that are commercially available (Hsu et al., 2005; Boersema et al., 2008).

Reductive dimethylation is routinely used for biomarker identification and quantitation. Varicose vein biomarkers have recently been profiled combining reductive dimethylation chemical tagging with HILIC-based fractionation and MS analysis using an LTQ Orbitrap (Huang et al., 2015). Varicose vein tissue analysis yielded 12 downregulated proteins as compared with healthy vein tissue. Of these 12, eight were glycolysis-related proteins that can serve as important biomarkers for the inhibition of the glycolysis pathway and the eventual dysfunction of the smooth muscle lining in the vein.

#### 12.2.4 Chemical Labeling (Isobaric)

Isobaric chemical labeling strategies take advantage of advanced MS/MS techniques to make quantitative measurements of peptides and proteins using chemical label reporter ions. Like nonisobaric approaches, multiple chemical functionalities are accessible for labeling with isobaric tags though most focus on amine labeling as targeting this group provides a near global representation of peptides from a protein digestion. A significant challenge in developing isobaric tags lies in the need to create a series of isobaric tags that fragment at a predetermined linkage yielding reporter ions of unique isotopic composition. Extending this challenge is the desire to create isobaric tags that can be produced in a cost-efficient manner and also label peptides with high fidelity. Each isobaric tag contains three essential elements: a chemical functional group reactive tag, an isotopically incorporated reporter, and an isotopically incorporated balance group to bring the total isotope count for each labeling reagent to the same count (Figure 12.3).

Isobaric tags for relative and absolute quantitation (iTRAQ) is an important isobaric labeling technique that was first introduced in 2004 (Ross et al., 2004) and has since been commercialized (Glen et al., 2010). The iTRAQ reagent uses an amine-reactive *N*-hydroxysuccinimide

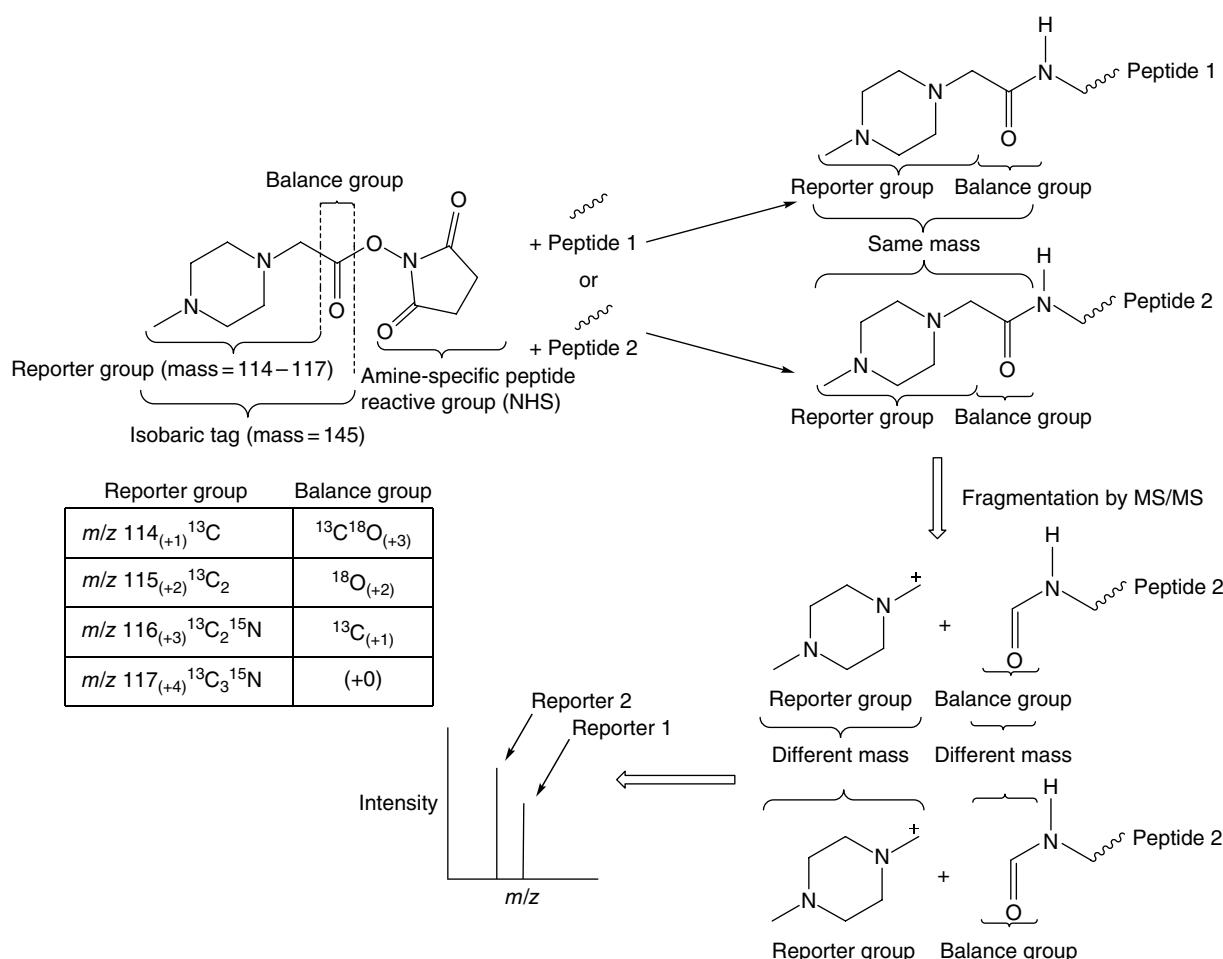


Figure 12.3 iTRAQ 4-plex reagent workflow. NHS, N-hydroxysuccinimide.

group to globally label primary amine groups. An *N*-methylpiperazine group with differentially incorporated isotopes serves as the reporter ion, and a linkage between this group and the *N*-hydroxysuccinimide provides a site for further isotope incorporation to provide a complement of isobaric tags (Figure 12.3). Isotopes are incorporated into the reporter and balancing group via  $^{13}\text{C}$ ,  $^{15}\text{N}$ , and  $^{18}\text{O}$  atoms. Two different linker balancing group designs have allowed for the commercialization of both a 4-plex and an 8-plex version of the iTRAQ reagent, and many important biomarker studies have commenced with the use of these tags (Guo et al., 2015; Zhu et al., 2016).

One recent example examining the heterogeneity found in rheumatoid arthritis (RA) patients highlights the exploratory nature of biomarker quantitation through the use of these isobaric iTRAQ tagging reagents (Ortea et al., 2016). In an effort to better understand the origins of patient-specific drug responses, Ortea et al. used iTRAQ 8-plex labeling to elucidate biomarkers resulting from the physiological response to two

RA drugs, adalimumab and infliximab. Patients were classified into nonresponders or responders for each drug and biomarkers were sought that would help discriminate between these two groups. Serum samples were digested, iTRAQ labeled (four responders and four nonresponders per drug trial), and analyzed with nanoHPLC-MS/MS coupled to an Orbitrap Velos hybrid mass spectrometer. 264 proteins were identified in all samples, and nine of these proteins showed differences between responders and nonresponders after adalimumab treatment. Interestingly, the putative biomarkers of infliximab treatment (responder vs. nonresponder) were not shared among the adalimumab protein results, thus yielding a unique set of drug-specific biomarkers for RA treatment.

Another commonly employed isobaric reporter ion (MS/MS) biomarker technique is tandem mass tag (TMT) labeling. TMT reagents (Thompson et al., 2003), like iTRAQ reagents, can be used in multiplex format. Originally designed as 2- to 6-plex reagents, Gygi and coworkers spent considerable effort expanding the utility

of TMT reagents to include an 8-plexing reagent (McAlister et al., 2012). TMT 8-plexing uniquely takes advantage of the mass defect that results from the nuclear binding energy differences, <sup>15</sup>N substituted for <sup>14</sup>N or <sup>13</sup>C substituted for a <sup>12</sup>C, to create reporter ions of nearly identical mass, 6mDa *m/z* differences, which can be resolved using high-resolution MS instruments such as Orbitrap analyzers. In that seminal work, Gygi showed that the isobaric TMT isotopologs could efficiently quantify mouse brain and spleen (four replicates each) protein expression with results matching known standards (Su et al., 2004; Kislinger et al., 2006). In all, 941 proteins were identified and quantified, 835 of which were differentially expressed between the two different tissue types. Impressively, this same group has expanded the utility of TMT further to include an 18-plex set of reagents that when combined with the SILAC labeling technique provided a hyperplexing approach for analyzing 54 samples simultaneously (Everley et al., 2013).

Others have used the original isobaric TMT reagents, with reporter ions at *m/z* values ranging sequentially from 126 to 131, to investigate protein concentrations in numerous cell types. Original TMT labeling has been used to validate the proteome homogeneity of cells derived from differentially induced pluripotent stem cells (iPSC) (Trakarnsanga et al., 2014). Importantly, 1989 proteins were identified and quantified from the proteome of erythroid cells differentiated from three different iPSC lines. Only 1.9% of the 1989 proteins differed in level by fivefold or more between samples. This multiplexed proteome analysis allowed the authors to conclude that the proteome of erythroid cells produced from different iPSC lines is very similar to adult erythroid cells and might be suitable for transfusion therapy.

TMT reagents have found use in measuring protein glycosylation by exchanging the original *N*-hydroxysuccinimide group (amine targeting) for an aminoxy group (aldehyde targeting) (Hahne et al., 2012). The new set of reagents, termed aminoxyTMT, has been used to study *N*-glycans derived from human blood serum. In one such study, blood serum proteins from patients suffering from three different esophageal diseases were labeled with multiplex aminoxyTMT reagents and analyzed on an LTQ Orbitrap instrument following UHPLC purification (Zhou et al., 2016). Of the more than 40 different glycans that were simultaneously quantified, several distinct glycans were implicated as unique biomarkers for each of the three esophageal disease states. Work to validate these results is ongoing, but the expansive high-throughput analysis of a multiplexed complicated glycan mixture is noteworthy.

TMT and iTRAQ isobaric techniques both suffer from the interference of coisolated and cofragmenting peptide

ions that lead to ratio distortions of isotopically labeled peptides (Karp et al., 2010; Ting et al., 2011; Erickson Brian et al., 2015). Several methods of experimental design and bioinformatic manipulation have been developed to minimize or alleviate these ratio distortions. Much work has focused on selecting the desired MS<sup>2</sup> fragment ions for further collision-induced dissociation and quantification in the Orbitrap, MS<sup>3</sup> (Liu et al., 2016). These MS<sup>3</sup> approaches have generally led to a significant reduction in interference ions with the concomitant increase in sample preparation and processing time. Others have recently focused on the use of internal MS standards to calibrate the ratio of TMT or iTRAQ reporter ions, thus mitigating the influence of interfering ions. Examples using three- or six-standard proteins (Ahrne et al., 2016; Paulo et al., 2016) have been reported, and in each case mathematical modeling of ratio compression increased quantification accuracy with sensitivity improvements of up to 40% in some cases.

The large expense associated with the preparation of the intricate isobaric reagents used in TMT and iTRAQ labeling has caused some to investigate other less expensive isobaric reagents. While working with dimethylated peptides, Li and coworkers recognized that an N-terminus dimethyl-Leu (DiLeu) residue produced a very intense a1 ion and reasoned that an isobaric reagent based on this scaffold could be both economical and provide for enhanced detection and quantitation by MS<sup>2</sup> (Xiang et al., 2010). A series of four isobaric DiLeu reagents were constructed using mixtures of <sup>2</sup>H, <sup>13</sup>C, <sup>15</sup>N, and <sup>18</sup>O that when analyzed by MS<sup>2</sup> yielded four unique reporter ions (*m/z* = 118.1, 117.1, 116.1, 115.1). Importantly, all four reagents can be synthesized in one or two steps from commercial available isotopic leucine reagents, sodium cyanoborodeuteride, and heavy water. The DiLeu reagents are made nitrogen reactive by the installation of a triazine ester using 4-(4, 6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride in the presence of an *N*-methylmorpholine base. The overall cost of the 4-plex DiLeu labels for 100 µg of protein is estimated at 10 dollars, whereas the equivalent amount of iTRAQ reagents is estimated to cost 20 times as much.

In that seminal work, Li et al. demonstrated that DiLeu-labeled tryptic BSA peptides yielded a sequence coverage of approximately 43%, the same result that iTRAQ labeling provided. The DiLeu tryptic peptides also showed better fragmentation and higher reporter ion intensities than iTRAQ. A neuropeptide analysis of the pericardial organ of four blue crabs was performed in a multiplexed format, yielding a total of 131 MS<sup>2</sup> spectra in a single LC-MS<sup>2</sup> run. Up to twofold expression differences were noted for several neuropeptides found in the

four blue crab specimens, suggesting individual animal variability arising from different physiological states.

An 8-plex version of the DiLeu reagent was soon introduced (Frost et al., 2015b). This larger set of multiplex reagents takes advantage of a larger than carbonyl (C–O), but also inexpensive, alanine balancing group for the incorporation of a greater number of isotopes than previously possible with the 4-plex design. Even with the added size of the alanine isotopes, the 8-plex reagents provide a cost of labeling of \$15 for 100 µg of digest. The resulting 8-plex DiLeu label adds a mass of 220.2 Da to labeled peptides as opposed to the mass of 145.1 Da that was used in the original 4-plex DiLeu approach. Reporter ion masses range from *m/z* = 115 to 122.

The 8-plex DiLeu labeling reagents were tested for fragmentation efficiency, chromatographic retention, quantitative accuracy, and dynamic range of a yeast lysate digest using a Q-Exactive Orbitrap mass spectrometer. Fragmentation efficiency, quantitative accuracy, and dynamic range were all shown to be similar to the 4-plex DiLeu reagents as approximately 1000 protein groups were accurately quantified for protein ratios ranging from 1:1 to 10:1. Chromatographic retention differences did arise for the deuterium containing reagents as reversed-phase chromatography is known to discriminate hydrophobic residues containing the deuterium isotope such as those found on the alanine balancing group in reagents 115–118 (Zhang et al., 2001). It is necessary in 8-plex DiLeu labeling to normalize relative quantitation of reagents 115–118 independently from reagents 119 to 122 due to this chromatographic shift.

The recently described 12-plex DiLeu labeling extends the multiplexing capability of DiLeu reagents without an increase in label complexity. This is achieved by using a high-resolution high-energy C-trap dissociation Orbitrap mass spectrometer and exploiting mass defect differences (~6 mDa) that arise from differential incorporation of <sup>12</sup>C/<sup>13</sup>C, <sup>14</sup>N/<sup>15</sup>N, and <sup>1</sup>H/<sup>2</sup>H as described previously for TMT 8-plexing (Frost et al., 2015a). Sharing structural similarities with 4-Plex DiLeu, the 12-plex DiLeu reagents are synthesized in the same efficient (80% yield) two-step synthetic process. A complex mixture of *S. cerevisiae* lysate tryptic peptides were labeled in triplicate with each of the 12 DiLeu reagents in ratios of 16:8:4:2:1:10:10:1:2:4:8:16. Approximately 700 protein groups were identified across this large dynamic range with median quantitative ratios within 10% of the expected values, and coefficients of variation averaged 11.5%. The 12-plex DiLeu labeling scheme was demonstrated to be both accurate and precise over a large dynamic range, making 12-plex DiLeu labeling an

attractive and economical multiplexing approach for the high-throughput quantitation of biomarkers.

## 12.3 Conclusions

Recent advances in MS instrumentation and the novel design of new isotopic reagents have allowed a wide variety of biomarker discovery tools to be developed and even commercialized. The broad-based use of these techniques has allowed greater insight into biological systems and the biomarkers that control them. As isotopic labeling techniques garner greater support, more research efforts have been put forth to increase the quality of isotopic labeling approaches. Essential to the effective design of new isotopic labeling approaches should be the desire to reach the following goals: (i) the use of cost-effective isotopes in the construction of labeling isoptomers; (ii) the ability to target and label numerous biomarker classes (i.e., amine-, carboxylic acid-, alcohol-containing, etc.) with quantitative efficiency; (iii) a scaffold that is capable of incorporating many unique isotope compositions such that multiplexing approaches are possible; (iv) a simple and predictable isotopic signature peak in either the MS<sup>1</sup> or MS<sup>2</sup> spectrum; and (v) a charge enhancement functionality to simplify detection of biomarkers.

The research techniques highlighted in this chapter showcase many of the important early biomarker isotopic labeling techniques. Each technique delivers in one or more of the five essential areas of development as mentioned above. Future work will undoubtedly be focused on optimizing these traits and also on combining isotopic labeling approaches to create a more global detection for all relevant biomarkers in a biological system. Mixing approaches also introduces the possibility of creating mixed multiplexed approaches wherein 20 or more samples could be quantified simultaneously. The use of high-resolution mass spectrometers and mass defect differences in isotope atomic masses will allow these mixed multiplexed approaches to expand. Two areas of advancement that will certainly enable growth in isotopic labeling approaches include advancements in chromatographic separations and bioinformatics. Biomarker sample complexity will be greatly diminished with improvements in chromatographic separations, and the information gleaned from the rich data provided by mass spectrometers can only be efficiently mined through the judicious use of bioinformatics suites. Creative solutions to biomarker discovery and quantitation will continue to be at the forefront of life science discoveries until a more holistic understanding of biological systems regulation is achieved through these efforts.

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## Part III

### Applications

## 13

### Targeted Quantification of Amino Acid Biomarkers Using LC-MS

Barry R. Jones, Raymond F. Biondolillo, and John E. Buckholz

*Q2 Solutions, Ithaca, NY, USA*

#### 13.1 Introduction

As the building blocks of proteins, amino acids support the diversity of life. They also exist in the body as individual molecules and individually play critical roles in many biochemical pathways. As such, quantitative measurement of amino acids in biological fluids can yield important information in disease identification and monitoring, treatment efficacy, and overall improvement of human health. In this chapter, we briefly discuss how the general structures of amino acids make them important to various biochemistries. We present some examples where measurement of amino acids gives insight into the function of a biochemical pathway and may provide an indication of disease. Ultimately, we examine analytical approaches to measure these molecules and offer guidance to ensure optimum assay characteristics such as accuracy, precision, and selectivity using methodologies involving LC-MS.

There are 20 common human amino acids and many more variations and metabolites. Nine amino acids are referred to as essential amino acids, meaning they cannot be synthesized in the body and so must be included in the diet. All contain a carboxylic acid and an amino group on the alpha carbon, which also carries varying side chains. Most are alpha amino acids, where the side chain exists at the position number 2 on a chiral carbon. Though both L- and D-isomers exist, L-isomers are far more abundant in biology. Amino acids are amphiprotic, as they have a basic amine group that can be protonated and possess positive charge and have a carboxylic acid group that can be deprotonated to possess negative charge. Both the amine group and carboxylic group are critical to the formation of linear sequences to build peptides and proteins. The chemical diversity of the side chains leads to the classification of amino acids with various chemical properties: acidic, basic, polar, hydrophobic, etc.

The side chains of amino acids are important to protein structure and stability. Both aliphatic nonpolar and aromatic side chains tend to be hydrophobic, which aggregate in an aqueous environment, giving peptides internal stability. Conversely, hydrophilic side chains are more apt to be exposed to aqueous conditions and thus are more readily available for interaction with other molecules. Cysteine is a polar amino acid with a thiol group in its side chain. Often, a pair of cysteines will form a disulfide bond, which can play a major role in three-dimensional protein structure. The amino acids with ionizable side chains can act as enzymatic active sites. For example, the ε-amino group of lysine is the target for ubiquitin conjugation during protein degradation.

In addition to protein synthesis, free amino acids are important molecules in metabolic pathways. Amino acids are critical in nitrogen metabolism and excretion of ammonia from the body. Aminotransferase enzymes remove the amino group from a specific amino acid and transfer it to an α-keto acid. A common reaction of this type is the transfer of an amino group to α-ketoglutarate to form the amino acid glutamate. Glutamate then enters the liver where the amino group is removed and converted to urea for excretion. Glutamine is also vital to the removal of ammonia. In most tissues of the body, glutamine synthetase catalyzes the addition of a free amino group to glutamate, resulting in glutamine. Glutamine then travels in the bloodstream to the liver where the enzyme glutaminase converts glutamine back to glutamate and the ammonia enters the urea cycle.

Amino acids can also be funneled into metabolic pathways to be used as an energy source. Amino acids can be either ketogenic or glucogenic. Ketogenic amino acids are converted to acetyl-CoA or acetoacetyl-CoA and are moved to the liver where they become ketone bodies. Glucogenic amino acids are those that can be used to create glucose and glycogen. First, glucogenic amino acids are directly converted either to pyruvate or to

another tricarboxylic acid (TCA) cycle intermediate such as  $\alpha$ -ketoglutarate, succinyl-CoA, fumarate, or oxaloacetate. From there, these intermediates can enter into the gluconeogenesis pathway. The only amino acids that are not glucogenic are lysine and leucine.

In contrast to most amino acids, branched-chain amino acids (BCAAs) (valine, leucine, and isoleucine) are usually metabolized outside of the liver (e.g., in the muscle, kidney, and brain tissue). BCAAs are converted to their  $\alpha$ -keto acid derivative via an aminotransferase not present in the liver. These  $\alpha$ -keto acids are further catabolized to their acyl-CoA derivative via the branched-chain  $\alpha$ -keto acid dehydrogenase complex, which acts on all three BCAAs. From there, leucine is converted to acetyl-CoA and is moved to the liver for ketone body production. Isoleucine and valine are both ketogenic and glucogenic as both can be converted to succinyl-CoA.

Amino acids also serve as precursors for neurotransmitters, many of which contain an amine group. Tyrosine is a precursor for dopamine, norepinephrine, and epinephrine; decarboxylation of glutamate and histidine

results in gamma-aminobutyric acid (GABA) and histamine, respectively; and tryptophan can be metabolized to serotonin (Nelson and Cox, 2005; Berg et al., 2012).

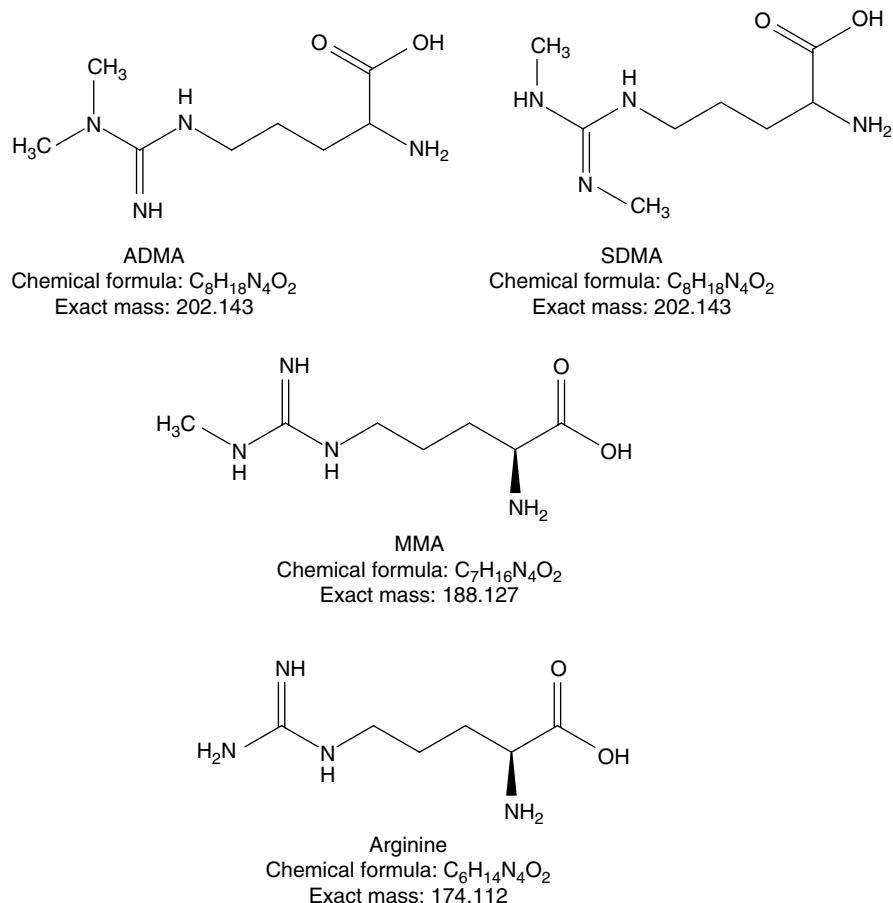
## 13.2 Amino Acids as Biomarkers

As described above, amino acids play critical roles in many biological functions. Reference ranges for amino acids have been developed from extensive historical research and data from many quantitative methodologies. Normal relevant biological functions are suggested with circulating amino acid levels within “normal” ranges, and these ranges vary depending on age and bodily fluid. Examples of amino acid reference ranges in plasma are shown in Table 13.1. As levels outside of these ranges may be indicative of disease, amino acid measurement can be a useful diagnostic tool. Whereas a biomarker for some diseases may consist of one or two amino acid profiles, it is a complex landscape due to the ubiquity of amino acid biochemistry, and elevation of one or two amino acids alone may point to more than one potential malady.

**Table 13.1** Reference ranges for normal amino acid concentrations in human plasma (Scriven et al., 2001; Blau et al., 2003; Nyhan et al., 2005).

Normal range of endogenous amino acids in human plasma ( $\mu\text{g/mL}$ )				
Amino acid	<1 month	1–23 months	2–17 years	Adults ( $\geq 18$ years)
Alanine	7–40	11–47	14–43	18–43
Arginine	2–21	5–23	6–19	7–64
Asparagine	2–9	3–10	3–9	4–8
Aspartic acid	0–3	0–2	0–1	0–1
Cysteine	N/A	0–4	0–4	0–12
Glutamic acid	8–41	5–27	1–16	1–14
Glutamine	35–174	44–213	59–135	63–109
Glycine	10–31	8–29	10–26	9–24
Histidine	6–22	7–19	8–18	9–17
Isoleucine	2–12	1–14	4–13	4–13
Leucine	3–23	6–24	9–23	10–24
Lysine	10–33	10–38	14–34	17–34
Methionine	2–7	2–7	2–6	2–5
Phenylalanine	5–13	5–15	6–14	7–12
Proline	10–43	12–40	11–40	12–44
Serine	9–25	9–22	9–19	7–15
Threonine	7–47	5–51	7–23	8–24
Tryptophan	3–17	3–19	6–19	8–19
Tyrosine	6–29	4–23	6–20	7–17
Valine	7–29	10–41	15–36	15–37

**Figure 13.1** The structures of symmetric dimethylarginine (SDMA), asymmetric dimethylarginine (ADMA), monomethylarginine (MMA), and free arginine.



Furthermore, lifestyle and nutrition differences alone may lead to elevated or lowered levels of amino acids. With proper understanding of the surrounding biology, complex interplays of different circulating amino acid fluctuations may make for good disease signature. This drives the prevalence of amino acid panel assays.

Below we list some examples where amino acids have therapeutic or diagnostic value. This is only a sampling of applications for relevant amino acid biomarkers and is not comprehensive, meant to underline the importance of accurate amino acid biomarker measurement in biological fluid and tissue as well as the complexity of the associated biochemistry.

### 13.2.1 Biomarker of Heart Failure

A characteristic of cardiovascular disease progression is the altered nitric oxide (NO) bioavailability. Studies suggesting that the synthesis of NO from L-arginine may be altered by the formation of a complex series of methylation pathways have led to reports of measurement methodologies for symmetric and asymmetric dimethylarginine (SDMA and ADMA, respectively), monomethylarginine

(MMA), and L-arginine (Figure 13.1). MMA and its methylated metabolite ADMA have been identified as potent inhibitors of endogenous nitric oxide synthase (NOS). Accumulation of ADMA in particular has emerged as a novel cardiovascular risk biomarker associated with type 2 diabetes mellitus, coronary artery disease, end-stage renal disease, and heart failure. It has been shown that intravenous administration of low-dose ADMA in healthy patients had a negative effect on blood pressure and cardiac function. Taken together with observations that patients with heart failure had increased circulating levels of ADMA compared with healthy controls, methylated arginines and ADMA in particular may be a useful biomarker for heart disease (Wilson Tang et al., 2008).

### 13.2.2 Citrulline as Biomarker of Intestinal Failure

Citrulline has been used as a biomarker of intestinal failure due to enterocyte mass reduction (Crenn et al., 2008). Citrulline mainly comes from the conversion of glutamine in the enterocytes located in the middle and upper parts of the intestinal villi, and plasma levels can

indicate the health of the intestinal mucosa; a decrease is diagnostic of reduced enterocyte mass. Citrulline is a strong indication of acute cellular rejection (ACR) when monitored following small bowel transplantsations (SBT) in patients with irreversible intestinal failure or short bowel syndrome (Honsova et al., 2009).

In addition to the value of citrulline measurement as an indicator of intestinal functional mass, low levels are observed in critically ill people with sepsis and even lower in patients with acute respiratory distress syndrome (ARDS), suggesting its value as a biomarker. This is complicated biology due to the interplay between arginine and citrulline in NO synthesis and regulation by glutamine, which is also lowered in patients with sepsis. Regardless, it does appear that citrulline supplementation may also help critically ill people with ARDS (Cynober, 2013).

### 13.2.3 Oncological Biomarkers

The nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>)-dependent isocitrate dehydrogenase genes IDH1 and IDH2 are mutated in the majority of low-grade gliomas and secondary glioblastoma multiforme (GBM). Mutated IDH1 and IDH2 lose normal catalytic activity and gain the ability to catalyze the reduction of alpha-ketoglutarate ( $\alpha$ -KG) to produce the D-enantiomer of 2-hydroxyglutarate (D-2HG), resulting in an accumulation of D-2HG that may point to gliomas and acute myeloid leukemia (AML). In some cases D-2HG can accumulate to the low millimolar concentrations. Such a large fold factor of difference between disease and healthy makes measurement of D-2HG an attractive biomarker for these oncological applications. However, the precise mechanism of action to support D-2HG as an oncometabolite is not known. The only structural difference between D-2HG and  $\alpha$ -KG is that the oxygen atom at the C-2 position in  $\alpha$ -KG is replaced by a hydroxyl group for D-2HG (Figure 13.2). Some researchers point to this structural similarity as evidence for the possibility that D-2HG may act as a competitive inhibitor of  $\alpha$ -KG-dependent dioxygenases (Xu et al., 2011).

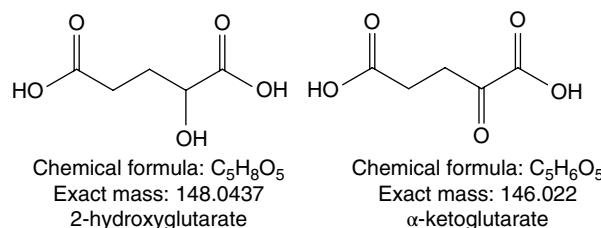


Figure 13.2 The structures of 2-hydroxyglutarate and  $\alpha$ -ketoglutarate.

### 13.2.4 Branched-Chain Amino Acids in Diabetes and Cancer

BCAAs consist of leucine, isoleucine, and valine. BCAAs are critical for the synthesis of neurotransmitters and proteins. They are essential amino acids and therefore are not synthesized in the body so they need to be obtained from diet. Measurement of BCAAs outside of a carefully controlled clinical setting is difficult as levels are impacted not only by diet but also by a variety of clinical states. BCAAs have therapeutic value as they may help spare lean body muscle in times of weight loss, promote wound healing, and have benefits during renal and liver disease. Use of BCAAs alone as biomarkers is problematic again due to the complexity of the involved pathways, and changes in measured concentrations may or may not be meaningful. Measurement of BCAAs in conjunction with other assessments may be of more use and lead to a better understanding of their roles and functions (Tom and Nair, 2006).

Cancer and diabetes cause large metabolic perturbations that affect the entire body. BCAAs seem to be among the most perturbed of the amino acids, and monitoring them could be of enormous clinical value due to their critical roles. The challenge in using BCAAs as effective biomarkers is that depending on health, disease state, or tumor location, the endogenous levels can be affected differently. Similar to the biological mechanisms causing BCAAs to be instrumental in muscle development, they are also critical for tumor development. The effective use of BCAAs as biomarkers will be in context with other metabolic information and may be useful in predicting both cancer and diabetes in early stages. In addition to these metabolic processes, studies have also shown activity of BCAAs in cell signaling pathways supporting protein synthesis and cell growth. Abnormalities in metabolite profile could indicate irregularities in biological pathways that may drive insulin resistance and tumor progression in subjects (O'Connell, 2013).

### 13.2.5 Inborn Errors of Metabolism

Occasionally, inborn errors of amino acid metabolism lead to a disease state. Maple syrup urine disease, or branched-chain ketoaciduria, is a result of an inactive branched-chain  $\alpha$ -keto acid dehydrogenase complex causing an accumulation of the branched-chain  $\alpha$ -keto acids in blood and urine, giving the urine a distinct maple syrup odor. The majority of untreated patients die within first few months of life from “recurrent metabolic crisis” and neurologic deterioration unless treated with both aggressive therapeutic intervention and long-term management of diet (Ævarsson et al., 2000; Chuang et al., 2004).

Inborn metabolism errors of lysine, arginine, and ornithine are not prevalent in the population. Because of this, it is hard to identify safety limits of concentration and to use them as biomarkers. These amino acids are linked to the urea cycle. Hyperlysinemia is the result of a deficiency in 2-aminoacidic semialdehyde synthase. This catabolic error leads to increased levels of lysine in plasma and cerebrospinal fluid (CSF). Interestingly, about half of the patients with increased levels of lysine show no symptoms, while the other half exhibit mental retardation, seizures, muscular hypotonia, and spasticity (Saudubray and Rabier, 2007).

### 13.2.6 Biomarker of Phenylketonuria (PKU)

Phenylketonuria (PKU) is another example of a disease caused by an error in amino acid metabolism. Instead of being catabolized to tyrosine, phenylalanine accumulates in the blood due to a deficiency in the enzyme phenylalanine hydroxylase. Recognized as early as 1934, the disorder leads to developmental problems in infants. Unfortunately the issue was only discovered postmortem and the need for a diagnostic test was recognized. In 1963 Robert Guthrie had developed a simple bacterial inhibition test to screen for the presence of this disorder a short time after birth and before harm had come to the infant. The test was performed by doing a heel prick and spotting onto a preprinted collection card from an infant between days 2 and 5 following birth. A small disc was then punched from the center of the card and applied to an agar plate. Overnight bacterial growth on the medium was a positive indication of PKU. In 1985 all 50 states had a mandatory requirement for the screening procedure. In 1990 David Millington had developed a screening procedure by measuring phenylalanine and tyrosine using tandem mass spectrometry (MS/MS) instead of the bacterial inhibition test (Laboratories AoPH, 2013). Measurement of these amino acids by fluorometric methods may lead to false positives, and measurement by mass spectrometry (MS)-based techniques have increased accuracy of this biomarker measurement. In the case for PKU, which is associated with the decreased conversion of phenylalanine to tyrosine, a relevant biomarker assay to measure ratio of the two components can be used, though it may complicate assay control design (Chace et al., 1993).

### 13.2.7 Amino Acid Supplementation

Dietary interventions with specific amino acids appear to be mostly capable of stimulating protein synthesis. Most work has been done on healthy patients though there is hope that amino acid supplements will also help in the disease state too. Supplementation studies have been

done for cancer, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) (Jonker et al., 2012).

Arginine serves as a precursor for the production of NO. NOS converts arginine into citrulline, a non-proteinogenic amino acid, and NO. Arginine has thus been used as a nutritional supplement as NO production is important to cardiac health by relaxing cardiac tissue. Use of citrulline alone for nutritional supplementation has been shown to be more effective at increasing protein synthesis than arginine supplements (Schwedhelm et al., 2008).

## 13.3 Methods of Measurement

Commercially available colorimetric or fluorometric kits can be used for quantitative measurement of amino acids; however these represent a total measurement of amino acid classes rather than individual amino acid molecules. Fluorometric measurement (done at  $\text{Ex}/\text{Em} = 535/590$ ) is roughly 10 $\times$  more sensitive than colorimetric measurement ( $\text{OD}_{570\text{nm}}$ ) (Abcam, 2014).

For the measurement of individual amino acids, a mechanism of separation is required. Based on each amino acid's unique side chain, different characteristics of the side-chain moieties can be leveraged to discriminate one amino acid from another via liquid chromatography (LC). With sufficient separation, and typically with chemical derivatization to improve UV absorption, LC-UV techniques can be employed for quantitative measurement. For underivatized amino acids, there are few functional groups capable of absorbing UV light. Amino acids absorb UV light much better when derivatized—typical reagents include *o*-phthalaldehyde, phenyl isothiocyanate (PITC), fluorescamine, and dansyl chloride. This is also a good way to improve retention on reversed-phase columns for LC (Shimadzu, 2016).

Modern methodologies favor the use of MS. Multianalyte MS/MS screening from a single blood spot has revolutionized the process of newborn screening. Prior to this, each disorder had its own test, often requiring urine (which may not be readily available at birth) or larger amounts of blood for multiple stick tests. Also, quick turnaround is critical when dealing with newborns, so in addition to the minimally invasive nature of the tests, they are extremely fast and offer almost real-time feedback. This benefits sample throughput per instrument and, owing to the quality of the results, yields far fewer false positives. The methodology is readily expandable with modern instrumentation. This work can be done by flow injection analysis (FIA) with no chromatographic separation needed. Flow rates are generally  $<50\text{ }\mu\text{L/min}$ , often even lower between 10 and  $20\text{ }\mu\text{L/min}$ . The addition of a new analyte does not

require development of a whole new assay but is an addition to the current method (Chace et al., 2003).

Analysis by LC-MS/MS with triple quadrupole instruments allows great advantage over other analysis methods with improved selectivity due to combination of chromatographic separation, separation of molecular ion in first quadrupole, and separation of product ion in third quadrupole after fragmentation in collision cell (Q2). The product ions generated from CID of simple amino acids can be quite interesting, with cyclization and other gas-phase rearrangements possible. Low molecular weight product ions can challenge the typical mass calibration ranges of production spectrometers.

An advantage of chromatographic approaches to amino acid quantification is the ability to multiplex. It is possible to develop and validate an LC-MS assay to measure large panels of amino acids. Challenges encountered here for LC-MS applications include the need to balance chromatographic resolution with duty cycle limitations in order to preserve sufficient peak sampling. For selected reaction monitoring (SRM) applications using triple quadrupole mass spectrometers, it may be necessary to schedule SRM transitions in time, depending on ability to chromatographically separate groups of amino acids from one another with sufficient resolution to segment the acquisition into time periods. Advances in scanning speeds with modern instrumentation ease these challenges. Also, application of high-resolution accurate mass spectrometry in full scan mode may offer an alternative to segmented analysis by monitoring the same spectral width throughout acquisition such that all analyte molecular ions are captured and their specific ion currents extracted post-acquisition for quantification.

The base structure and zwitterionic nature of amino acids are such that optimal chromatographic conditions may not favor simple reversed-phase columns when the side chain does not provide for such retention chemistries. Consider glycine, which is an inhibitory neurotransmitter and an important biomarker of neurological disorders such as schizophrenia (Ji et al., 2011). Glycine is the smallest possible amino acid, having only a hydrogen atom as side chain. Typically, either hydrophilic interaction liquid chromatography (HILIC) (Prinsen et al., 2016), chemical derivatization (Wilson et al., 2011; Song et al., 2012), or ion-pair reversed-phase LC (Dang et al., 2009) are used. Owing to the diversity of amino acid side-chain chemistries, deriving suitable chromatographic conditions for large panel assays can be a challenge.

### 13.3.1 LC-MS Considerations for Measurement of 2-Hydroxyglutarate

As noted earlier, 2HG is a useful oncological biomarker. Here, it is presented as an example to demonstrate the

LC-MS considerations when measuring amino acids as biomarkers. Also discussed previously, the L-isomer of endogenous amino acids are typically far more prevalent; however for 2HG both L- and D-isomers are endogenous in human blood, and IDH1/2 mutations can cause elevated level of the D-2HG enantiomer. Because endogenous L-2HG and D-2HG levels are generally on the same order in healthy individuals, an LC-MS measurement that does not discriminate between enantiomers is not sensitive to small changes in D-2HG. Where significant accumulation of the D-2HG enantiomer is of interest, the combined enantiomer response is likely adequate, and measurement of total 2HG is a common approach (Gross et al., 2010; Sellner et al., 2010; Wang et al., 2013; Saha et al., 2014). This simplifies the associated chromatographic approach, as HILIC or ion-pairing approaches may be suitable (Brown et al., 2011). Enantiomeric separation, however, gives the most information into 2HG-related metabolic disorders (Seijo-Martínez et al., 2005; Kranendijk et al., 2010; Cheng et al., 2015).

Dang et al. (2009) have used a total 2HG method for investigations into the role of 2HG as a marker for IDH1 mutations as a feature in primary human brain cancers. The methodology, unencumbered by the need to distinguish between enantiomers, utilizes an ion-pairing reagent to establish retention on reversed-phase LC. It is the author's experience that the ion-pairing reagent is critical for adequate retention of 2HG on any reversed-phase column chemistry without analyte derivatization. Tributylamine (10 mM) was used as an ion-pairing agent in the aqueous mobile phase with a gradient 50–95% mobile phase B (100% methanol) over 5 min. No derivatization was required, allowing for simple extraction procedure and rapid analysis on relatively short LC cycle time.

Cheng et al. argued (2015) that accurate diagnosis of 2HG-related metabolic diseases relies on measurement of the separate enantiomers. This separation cannot be done by using MS alone and so is rather a chromatographic challenge. Separation must leverage the different arrangement of substituents around the chiral carbon using either a chiral analytical column (Rashed et al., 2000) or a derivatization that enhances those differences. Cheng et al. used the derivatization approach involving N-(*p*-toluenesulfonyl)-L-phenylalanyl chloride (TSPC) to separately quantify L-2HG and D-2HG. Here, the derivatized molecules exhibit much improved retention on reversed-phase column, and they are sufficiently different such that adequate separation of the enantiomers is achieved. The ionization efficiency is also greatly improved. Further, the derivatized molecules elute at a point where there is a higher organic solvent ratio, which further enhances ionization efficiency. Derivatization thus allows for chromatographic separation

and greatly improves sensitivity of the analysis. This comes at the cost of sample preparation complexity and a considerable increase in run time.

## 13.4 Accuracy, Precision, Selectivity, and Stability Considerations

### 13.4.1 Accuracy

The main analytical challenge to quantification of endogenous molecules is the presence of the target analyte in the test fluid. Perhaps the gold standard approach toward such quantification is standard addition, where the reference compound is spiked into the biological matrix at various concentrations. Here, the instrument response from the spiked samples is plotted against the nominal spike concentration. The response function will have a nonzero  $y$ -intercept due to the endogenous presence of the target analyte. Interpolation to the  $x$ -axis represents the negative of the endogenous analyte concentration. Using this approach, matrix effects may be controlled, as the “calibration” is done in the sample being tested. However, it is an extrapolative technique that is inherently less precise than an interpolative design. It is also prohibitively time consuming in a high-throughput setting. Further, this approach requires a relatively large sample volume, which may not be available for precious matrices (e.g., CSF), in some preclinical species, in pediatric patients, or where many other tests are to be performed on the same fluid aliquot. Ideally we want to measure the endogenous analyte once, by an interpolative technique, with calibrators free of endogenous response.

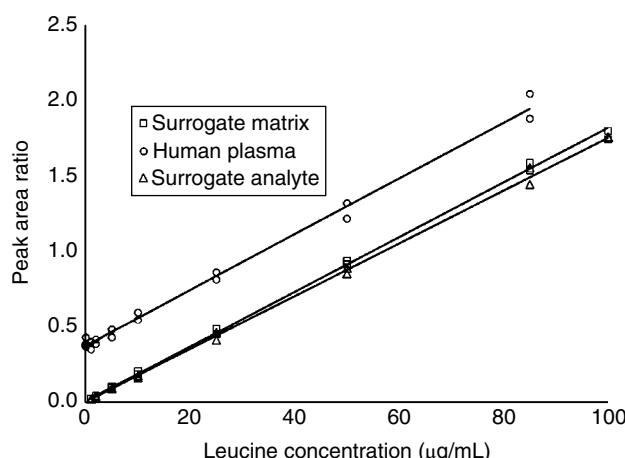
There are two main approaches to this for LC-MS applications: surrogate matrix and surrogate analyte. For surrogate matrix, authentic analyte reference standard is spiked into an analyte-free matrix for construction of calibration curve. This can be a synthetic matrix (bovine serum albumin (BSA) in phosphate-buffered saline (PBS)), a stripped matrix, or matrix from an alternative species (most common for polypeptides, where sequence homology is not conserved across species). Alternatively, we can leverage the ability of the LC-MS system to discriminate between isotopes via the surrogate analyte approach, in which a stable isotope-labeled (SIL) version of the target analyte is used to prepare calibrators in the authentic matrix, while a second SIL analog is used as internal standard (IS).

#### 13.4.1.1 Accuracy: Surrogate Matrix

As the physicochemical properties of the surrogate matrix may be different from the biological (authentic) matrix, care must be taken to ensure accuracy of the

sample measurements. Normalization of matrix effects between matrices by use of SIL-IS is highly recommended for these assays. For the surrogate matrix calibrators to accurately interpolate the endogenous concentrations in biological matrix, we must show that the slope of the surrogate matrix response function is equivalent to that in the biological matrix. Rather than using a straight comparison of slopes as indication of surrogate matrix acceptability, a method of comparison using the calculated endogenous concentration from standard addition and from replicate measurements interpolated from the surrogate matrix curve may be employed (Jones et al., 2012). As the extrapolation of the linear fit from the responses of spiked biological matrix to the  $x$ -intercept is one measurement of the endogenous analyte concentration, the interpolation of responses from unfortified biological matrix extracts by the surrogate matrix curve is another independent measurement. Both are derived as a function of the slope of responses in the respective matrix, and so a comparison between these estimated values for the same pool of biological matrix is a manifestation of comparison of slopes. However, the advantage to this approach is that a calculation of percent difference between the two concentration measurements allows application of bioanalytical assay criteria for evaluation of acceptance. This is more meaningful to the bioanalytical scientist than a calculation of percent difference between slopes, as acceptance criteria are not easily derived and applied to the direct slope comparison.

Figure 13.3 shows the response function plots for L-leucine extracted from human plasma via protein precipitation with acetonitrile and measured by LC-MS on a triple quadrupole mass spectrometer. The observed nonzero  $y$ -axis intercept is due to the endogenous presence of L-leucine. It is also evident from the near-zero



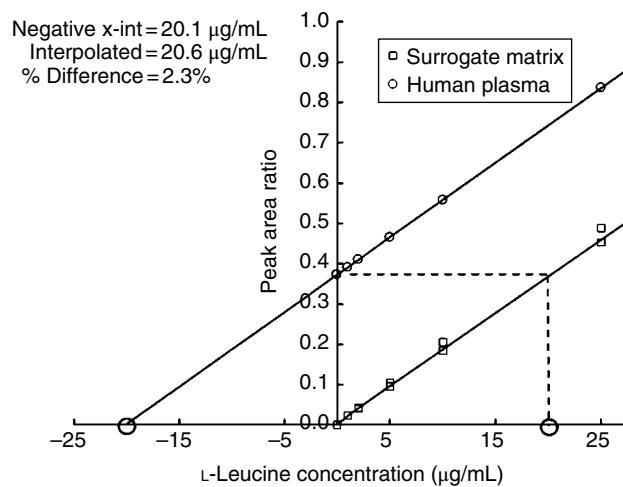
**Figure 13.3** The response functions of L-leucine extracted from human plasma and surrogate matrix (5% BSA in PBS).

intercept of the surrogate matrix response function fit that L-leucine is not present in 5% BSA in PBS, the surrogate matrix for this assay.

Upon visual inspection of Figure 13.3, it appears that the fits of these two functions are parallel. Indeed, parallel response functions are key to accuracy, but visual inspection is prone to subjectivity. Figure 13.4 shows the comparison between endogenous L-leucine concentrations derived from the negative x-axis intercept via standard addition (extrapolated) and interpolation of the mean response of six replicate analyses from the surrogate matrix curve (interpolated). At 2.3%, the difference between the two measurements is well within typical acceptance criteria, and parallelism can be assured.

This comparison is an assessment of parallelism using one distinct set of coordinates on this plot. We can estimate the impact of this derived lack of agreement between the slopes of the two plots by calculating the percent relative error (%RE) or % Theoretical (%Theo) of the spiked biological matrix quality controls (QCs). In this calculation ( $\%RE = 100 \times (\text{Observed conc.} - \text{Total theoretical conc.}) / \text{Total theoretical conc.}$ ), the theoretical total L-leucine concentration is calculated by addition of spiked reference compound concentration to the endogenous L-leucine concentration. For the parallelism assessment, the endogenous L-leucine

concentration is derived from the standard addition analysis rather than the interpolated endogenous value. This analysis is shown in Table 13.2.



**Figure 13.4** The parallelism experiment for a single amino acid (L-leucine) in a 5-amino acid panel assay using the surrogate matrix approach. The extrapolated endogenous analyte concentration is determined to be 20.1 µg/mL, whereas the mean ( $n=6$ ) interpolated endogenous analyte concentration is measured at 20.6 µg/mL for a 2.3% difference. These results indicate parallel response functions.

**Table 13.2** Comparison of calculated quality control sample statistics for L-leucine when correcting for endogenous concentration derived in two ways.

	Plasma diluted with surrogate matrix			Plasma	Fortified plasma			
	10× dilution	5× dilution	2× dilution		Endogenous	Low	GM	Mid
<b>Using endogenous concentration interpolated from surrogate matrix curve to correct total theoretical L-leucine concentration</b>								
Theoretical total concentration (µg/mL)	20.6	20.6	20.6	20.6	23.6	35.6	70.6	101
Mean calculated concentration (µg/mL)	20.5	21.7	20.4	20.6	23.1	31.4	70.7	102
%CV	7.9	11.4	21.7	5.8	3.7	5.5	4.8	5.3
% Theoretical	99.5	105.3	99.0	N/A	97.9	88.2	100.1	101.0
<i>n</i>	6	6	6	6	6	6	6	6
<b>Using negative x-axis endogenous concentration to correct total theoretical L-leucine concentration</b>								
Theoretical total concentration (µg/mL)	20.1	20.1	20.1	20.1	23.1	35.1	70.1	101
Mean calculated concentration (µg/mL)	20.5	21.7	20.4	20.6	23.1	31.4	70.7	102
%CV	7.9	11.4	21.7	5.8	3.7	5.5	4.8	5.3
% Theoretical	102.0	108.0	101.5	102.5	100.0	89.5	100.9	101.0
<i>n</i>	6	6	6	6	6	6	6	6

For the top panel, the total theoretical L-leucine concentration is adjusted by using the endogenous concentration interpolated from the surrogate matrix curve ( $n=6$ ). For the bottom panel, the total theoretical L-leucine concentration is adjusted by using the endogenous concentration extrapolated from a fit of the biological matrix response function to the negative x-axis. The % theoretical values agree well between approaches, illustrating excellent parallelism between biological and surrogate matrix functions. Statistics are based on six replicate analyses at each concentration.

### 13.4.1.2 Accuracy: Surrogate Analyte

As bioanalytical scientists employing SIL analogs of our target molecule as IS, we are encouraged by the physicochemical similarities of the analyte and IS. We are typically not concerned or even investigate the response of an authentic analyte compared with the SIL version at equal nominal concentration. However, there is often a disparity between these. There are many potential sources of response imbalance—ionization differences, gas-phase hydrogen–deuterium exchange, inaccuracies of purity determination, and compression of natural isotope distribution are examples. While these differences may not affect a typical assay when the SIL analog is used as IS, there is impact to accuracy when employing an SIL analog as a surrogate analyte.

Irrespective of the source of response imbalance, the effect can be characterized and the responses may be balanced. It is the authors' preference that this should be done experimentally and the imbalance corrected by attenuation of the more responsive transition prior to data acquisition. Once the responses have been balanced within specified criteria, parallel response functions are generally assured, as response imbalance is the likely source of slope difference between surrogate and authentic analytes. Within the assay design, all calibrators, blanks, QC<sub>s</sub>, and study samples are prepared in the biological matrix. Thus, the surrogate analyte method affords the benefits of interpolative quantification and avoids the effects of differing matrices encountered in surrogate matrix assays.

As with surrogate matrix approach, a similar comparison parallelism experiment for L-leucine can be performed for the surrogate analyte curve. At 8.6%, the difference between the extrapolated and interpolated measurements is well within typical acceptance criteria, and parallelism can be assured here as well. Using standard addition, surrogate matrix, and surrogate analyte, similar calculated concentrations of L-leucine in the same human plasma sample are generated.

The surrogate analyte approach does have disadvantages compared with surrogate matrix. When an SIL-IS is used for each analyte, there are three transitions required per analyte (authentic and surrogate analytes and IS), which impacts duty cycle and may be a concern especially for panel assays. Also, the obtained data must be manipulated prior to incorporation into a laboratory information management system (LIMS), as the relevant analyte transition is different for calibrators versus QC<sub>s</sub> and samples—data from three SRM transitions are acquired, yet information from only two are used for any one sample. Finally, as surrogate analyte assays are performed with biological control matrix, we do not have the luxury of diluting samples with responses that are above the quantitation limit (AQL) with an authentic

analyte-free matrix without additional experiments to qualify the use of a surrogate matrix. Back-calculation of AQL samples diluted with biological matrix must account for the endogenous presence of the analyte. This complicates dilution repeat analysis and is exacerbated with increasing endogenous level of authentic analyte in the control matrix. For surrogate analyte methods, it is beneficial to understand the expected range of target analyte concentration in healthy subjects, in disease state subjects, and after treatment with therapeutic, depending on the application, such that dilutions are avoided.

For surrogate analyte approaches requiring response balance and therefore signal detuning in SRM, the attenuation is generally done by adjusting collision energy toward a de-optimized fragment generation. Signal profiles across collision energy space are typically Gaussian and gradual, such that reproducibly attenuated response can be achieved with ability to fine-tune to be within a narrow acceptance criterion. For HRMS surrogate analyte methods using selected ion monitoring (SIM) acquisition, this is not an option. Here, alternative modes of signal attenuation may be employed, or an experimentally determined response factor may be applied to generated data without normalization of instrument responses pre-acquisition. Alternatively, the selectivity advantages of HRMS may be leveraged while including the fragmentation process by acquisition in an MS<sup>2</sup> mode. With MS/MS acquisition, response balance via attenuation of collision energy is again possible.

### 13.4.1.3 Surrogate Matrix/Analyte Considerations for Multiplexed Amino Acid Assays

Either surrogate matrix or surrogate analyte approaches are suitable for amino acid quantification. The relatively high concentration of endogenous amino acids tends to allow for effective dilution during extraction, which helps to normalize potential matrix effect differences between surrogate and biological matrices when using the surrogate matrix approach. Indeed, common extraction approach for serum or plasma is a protein precipitation followed by dilution of the supernatant. However this is not universally the case, and lack of parallelism of an amino acid analyte can be observed. To remedy this, extraction, LC, and MS parameters can be adjusted to optimize parallelism. As the number of analytes increases toward large panels, the method development efforts to align parallelism for all analytes under one set of extraction conditions and limited LC and MS options may be challenging. The surrogate analyte approach offers an advantage to large panels, as parallelism is essentially a function of response balance and can be achieved for all analytes without redevelopment. This comes with a cost,

as the complexity of assay management for a surrogate analyte panel method becomes much more complex compared with a surrogate matrix method due to the extra reference compounds and additional work required for data manipulation. The surrogate matrix approach for large panels is forgiving from a calibration range perspective—if one of the amino acid concentrations in a study sample is above the validated range, that sample can be diluted to within range with analyte-free diluent. In comparison, getting the ranges right is more critical for the surrogate analyte approach without such a clean diluent. The biological variability of many amino acids is easily within the typical linear range of LC-MS methodologies, so setting the appropriate assay range is generally feasible.

Because of the high endogenous abundance of amino acids such that interference or suppression from other endogenous analytes is minimal compared with low abundance molecules, the authors favor the surrogate matrix approach for amino acid panel assays. Parallelism is generally not a struggle due to the minimization of matrix effects through sample dilution during sample preparation procedures, such that the ultimate composition of biological surrogate matrices is made similar prior to the extraction step, helping minimize the differences in matrix effects. The assay design is simplified compared to a surrogate analyte method where the number of reference compounds and transitions are increased and manual manipulation of data output is currently required. However, either approach is valid.

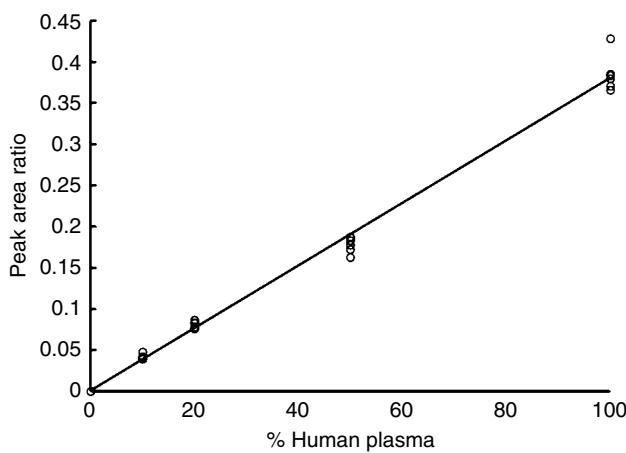
#### 13.4.2 Precision

An essential characteristic of quantitative biomarker assays is precision. The importance of assay precision is paramount particularly when the fold factor with disease state or treatment is small. In the batch-mode acquisition paradigm of bioanalysis, we must demonstrate that our assays are reproducible within and between experimental batches. It is the authors' practice to maintain a sufficiently large single pool of biological matrix to use in method development, method validation, and sample analysis modes. The calculated endogenous concentrations generated by replicate analyses within each batch are monitored (with considerations to analyte stability) for reproducibility, with intra- and inter-run precision measured against the assay needs and acceptance criteria. This practice is carried forth into sample analysis, with care to properly "bridge" the assay to new qualified biological matrix pools when volume or stability limitations make it necessary. This is considered a "longitudinal QC," the importance of which has been recently emphasized (Lowes and Ackermann, 2016).

#### 13.4.3 Selectivity

Selectivity is an important assay characteristic for low molecular weight biomarkers in biological matrices, owing to the multitude of similar circulating species. Because of the generally high concentration of the primary amino acids compared with less abundant endogenous molecules, the additional selectivity advantage afforded by SRM is not so critical. However, the inherent superiority of LC-MS selectivity is a valuable advantage for measurement of comparatively low abundance amino acid metabolites, such as methylated arginines (Brown et al., 2011; Andrade et al., 2015).

It is important to determine if the endogenous analyte can be diluted with surrogate matrix and recovered in a linear fashion when measured using surrogate matrix calibrators using authentic analyte. A graphical representation of the endogenous dilution linearity experiment for L-leucine in human plasma is shown in Figure 13.5. Here, good linearity was observed when plasma was diluted with surrogate matrix (5% BSA in PBS). Any observed nonlinearity can point to a selectivity issue in the biological matrix if the interfering species has a response function with a different slope compared with the target analyte (Buckholz et al., 2015). Nonlinearity on endogenous dilution can also arise if the reference compound is in a different form than the endogenous analyte. An example of this for small molecule analytes is for compounds that circulate as esterified versions of the base molecule, such as 4beta-hydroxycholesterol and cholesterol. Nonlinearity of endogenous dilution in these cases may be due to incomplete hydrolysis of esters, required prior to accurate measure of total analyte, which improves upon dilution. In this example, spiked parallelism results may show parallel response functions because the spiked reference is unesterified



**Figure 13.5** Endogenous dilution linearity for L-leucine in human plasma. The linearity and near-zero intercept suggest good assay selectivity and an appropriate surrogate matrix for quantitation.

and unaffected by hydrolysis efficiency, whereas endogenous dilution linearity cannot be successfully demonstrated (Jones et al., 2014). In the end, both parallelism and endogenous dilution linearity must be performed to “qualify” the surrogate matrix.

Implementation of high-resolution accurate mass spectrometry in regulated bioanalysis has revealed many opportunities for improved quantification of small and large molecule therapeutics and biomarkers. High mass resolution can provide a solution to the interferences observed in relatively low-resolution SRM approaches. For SRM assays that are sensitivity-limited due to high noise levels, HRMS has been shown to improve signal-to-noise levels via improved selectivity (Mulvana et al., 2015). The selectivity advantage is particularly attractive for targeted biomarker measurement, as the approach to demonstrating selectivity in a therapeutic pharmacokinetic (PK) assay, specifically analysis of control matrix blanks, cannot be done for endogenous analytes in the biological matrix. Endogenous dilution linearity as described may be a probe of endogenous analyte selectivity for an SRM assay, but it is limited and assumes disparate response functions between target analyte and interfering species upon dilution. High-resolution accurate mass analysis lends confidence that we are measuring what we think we are measuring, either in a confirmatory sense as supporting data for an SRM assay or as a platform for the validated biomarker assay itself.

#### 13.4.4 Stability

Generalization of the inherent ex vivo stability of amino acids will be avoided here due to the multitude of amino acids and metabolites monitored as biomarkers in diagnostics and drug development, although it is the authors' experience that the common amino acids are generally stable in biological matrices under usual conditions encountered in these labs. It is important to measure the stability of target analytes with the established methods and to probe such measurements as whole blood stability and benchtop, freeze/thaw, and long-term freezer storage stability in the unprocessed matrix (plasma, serum, urine, tissue homogenate). It is most relevant to measure the stability based on the calculated concentration of the endogenous analyte in the biological matrix rather than using a sample fortified with the reference material. It may be desirable to measure stability on spiked analyte to inform the assay management practices—how long bulk prepared spiked QC samples can be stored or how many times they may be freeze/thaw cycled and still maintain integrity, for example. We are measuring endogenous analyte in the biological matrix when we assay study samples, after all. Stability (or lack thereof) when measuring the spiked material may or

may not be reflective of the actual situation of the endogenous analyte. Measurement of stability based on endogenous analyte, however, requires that the stability experiments be performed as quickly as possible after collection of the control matrix used for the investigation. Such a stability measurement is necessarily a comparison between initial conditions and the stressed sample rather than a comparison to “nominal.”

## 13.5 Assay Design

The above approaches are useful principles to consider when developing and validating an amino acid biomarker assay. The ultimate assay design will depend on the specific amino acids being measured, as well as the associated biology. Consider, for example, an assay measuring two amino acids closely related in a biological pathway, perhaps with very similar structures, and where one analyte is a precursor of the other. We may expect one analyte to increase with disease state compared with normal (or with treatment compared with disease state) and the other to decrease in concentration. Measurement of 2-hydroxyglutarate and  $\alpha$ -ketoglutarate for oncological applications is an example. Another is phenylalanine and tyrosine measurement for PKU indications. For a panel amino acid assay with both up- and downregulation components, choosing assay approach and QCs could be challenging. One approach using the surrogate matrix technique is to use biological matrix diluted by several factors as QCs for the downregulated analyte and spiked biological matrix QCs for the upregulated analyte in the same method. Clearly, biomarker assays are more complex than PK assays, and it is far more difficult to apply prescribed bioanalytical method validation guidance. But we can let sound analytical principles and good biological knowledge guide our practices as we navigate proper assay controls for biomarker assays.

## 13.6 Conclusion

The authors of this chapter operate in the bioanalytical space supporting drug development, regulated by the Food and Drug Administration (FDA), European Medicines Agency (EMA), and others. For those in the clinical diagnostic space, practices and guidance (i.e., Clinical Laboratory Improvement Amendments) have already been established around biomarker quantitation. This is very much an active discussion as the regulators and bioanalytical practitioners debate how to regulate the use of biomarker assays to support new drug development.

A key aspect of this debate and a consideration for amino acid biomarker analysis is that of “commutability,” or the need to ensure that the measurement of a given analyte from one assay or lab matches that from another assay or lab. Given that interpretation of amino acid measurements in study samples will likely be referenced to a “normal” range, regardless of the singular importance of measuring the change with respect to drug treatment, the “commutability” of the assay should be an important consideration. Here, well-characterized amino acid reference materials are critical and fortunately readily available. For more complex molecules such as proteins, reference materials may not match the endogenous molecule, complicated by posttranslational modification, differing extents of glycosylation, etc. In these cases, some argue for the use of the endogenous molecule itself as calibrator using techniques

such as admixing (Grant and Hoofnagle, 2014) or reverse calibration (Wang et al., 2014). These calibration strategies require consideration from bioanalytical scientists and regulators, as they offer alternatives that address a main challenge for biomarker assay: limited availability of well-characterized reference materials. These advantages are maximum for large molecular targets, like peptides and proteins. For amino acids, the surrogate matrix and surrogate analyte approaches defined here in combination with well-characterized reference materials and the unparalleled selectivity of LC-MS methodologies should yield the absolute accuracy required for commutability and the precision required to measure potentially small changes in endogenous levels with treatment in order to provide quality biomarker data to support drug development strategies for combating disease.

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## 14

### Targeted Quantification of Peptide Biomarkers: A Case Study of Amyloid Peptides

Lieve Dillen, Marc De Meulder, and Tom Verhaeghe

*Pharmacokinetics-Dynamics and Metabolism, Regulated Development Bioanalysis,  
Janssen Research & Development, a Division of Janssen Pharmaceutica NV, Beerse, Belgium*

#### 14.1 Overview

Peptides and proteins are an important class of biomolecules. They play key roles in many critical functions in living cells and are involved in almost every physiological process. Both in fundamental and applied research, these molecules can provide insight into pathway signaling and the mechanism of action of therapeutics. The pharmaceutical industry has also recognized the potential of these molecules and incorporated them in their development programs as scaffolds for development of new drugs. In addition, the endogenous peptides/proteins are scrutinized as potential biomarkers to assess disease state or impact of a therapeutic intervention. Peptide/protein biomarker research has expanded tremendously over the last years with the focus mainly on the discovery and the biological validation of the proposed biomarkers. In analogy with proteomic investigations, peptidomics is defined as the comprehensive analysis of all peptides in a biological sample. It provides an excellent tool to examine differential abundance of peptides present in a biological system. Peptidomics studies the presence of endogenous peptides (often protein fragments) and has frequently been applied in the search for better disease biomarkers. Peptidomics-based efforts for biomarker discovery have delivered many promising candidates, but very few valid biomarkers have emerged from any of these studies (Dallas et al., 2015; Romanova and Sweedler, 2015; Schrader et al., 2015). In pivotal follow-up experiments, the quantitation of biomarkers has not always been executed with an appropriate analytical strategy to confirm the predictive value of the discovered biomarkers.

In this chapter the quantification of peptide biomarkers will be the major objective. The distinction between a peptide and a protein biomarker is not well defined and therefore only peptide molecules that can be analyzed with liquid chromatography–mass spectrometry (LC-MS),

as intact entities without need for further digestion into smaller fragments will be considered in this chapter. This limits the peptide length in practice to about 50 amino acids or to molecular weight (MW) below 10,000.

The standard assay for the quantification of these peptide biomarkers (as for protein biomarkers as well) in biological matrices still relies predominantly on immunoassays. These assays offer great sensitivity and throughput, but often suffer from lack of selectivity and specificity, not allowing differentiation between the peptide and its derivatives or degradation fragments (Hoofnagle and Wener, 2009). Often also limited dynamic ranges have been reported. Independent orthogonal techniques (such as LC-MS) can complement and support the scientific and analytical validity of these assays.

From an economic point of view, immunoassays for confirmation and validation of new putative biomarkers are not ideal for several reasons. First of all the preparation of appropriate affinity reagents, specific for each biomarker of interest, can be expensive and time consuming. They are typically not multiplexed to a large degree. Secondly, there has been interest to use separation-based methods, for example, LC-MS, to facilitate the confirmation and early validation of many putative biomarkers. These methods can be developed cost effectively and are easily transferrable to other labs. In addition, as a growing number of candidate protein and peptide biomarkers are discovered through proteomic or transcriptional profiling and no antibodies are available for absolute quantification in human clinical samples at an early stage, more and more targeted multiplexed LC-MS assays are developed. Ultrahigh performance liquid chromatography–mass spectrometry (UHPLC-MS) as well as UHPLC–tandem mass spectrometry (MS/MS) can be considered as an orthogonal technique to the immunoassay and can potentially address some of the shortcomings of immunoassays. Traditional selected

reaction monitoring (SRM) LC-MS assays, high-resolution full-scan or high-resolution multiple reaction monitoring (MRM) assays have been implemented (Van den Broek et al., 2010a, 2010b, 2010c; Rauh, 2012).

Results have been published from a large-scale inter-laboratory study on the validation of multiplexed LC-MS assays (Abbatiello et al., 2015). Eleven laboratories participated (using 13 different instruments) and provided data on the accuracy and precision of a multiplexed liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay with 125 peptides spiked into digested depleted human plasma. Sample preparation was centralized and blinded samples at four concentration levels were offered to the participating labs. The median coefficients of variation (CVs) were below 20% for 11 out of the 13 instruments, and 12 out of 13 instruments showed CVs of less than 15% for the median and higher concentration levels.

Exploration for discovery of new biomarkers often relies only on relative quantification, comparing healthy and diseased animal/patient samples or comparing therapeutically treated and placebo patient samples. At this stage the biomarker standards are not readily available. In a recent study (Sandanayake et al., 2014), biliary cancer-predictive peptide fragments were identified based on the hypothesis that cancer-specific exopeptidases exist that generate fragments from circulating proteins. Also published research identified peptides in urine of patients with rheumatoid arthritis with significantly increased or decreased concentrations (Stalmach et al., 2014). In these studies no labeling approach was included for differentiation. However, in the analysis of cellular dynamics and biological pathways, metabolic labeling by isotopes is generally recognized as a very useful tool (Shiio and Aebersold, 2006; Mann, 2014).

In this chapter the focus and the case will deal with absolute targeted quantification. With respect to absolute targeted quantification of peptides, the amount of published data is vast. Table 14.1 illustrates an attempt to give a flavor of the peptide biomarker LC-MS/MS assays published over the last 20 years. The smaller peptides are well represented. Neuropeptides like oxytocin, vasopressin, enkephalins, neuromedins, and substance P are analyzed in microdialysates, cerebrospinal fluid (CSF), or brain homogenates (Kusmierz et al., 1990; Lanckmans et al., 2007; Karbiwnyk et al., 2008; Xu et al., 2011; Zhang et al., 2011; Mabrouk and Kennedy, 2012; Maes et al., 2015).

In endocrinology, glucagon and insulin levels are important hormones for the regulation of blood glucose levels. Pathologies such as diabetes, pancreatic cancer, and some neuroendocrine tumors can be monitored through these markers. For both markers LC-MS/MS-based methods have been developed (Darby et al., 2001; Chambers et al., 2013; Howard et al., 2014). C-peptide

levels in urine have also been quantified with LC-MS methodology (Fierens et al., 2000, 2003; Rossing et al., 2005; Rogatsky et al., 2006a, 2006b; Stoyanov et al., 2011, 2013). Additional endocrinologic markers such as glucose-dependent insulinotropic peptides ( $\text{GIP}_{1-42}$  and  $\text{GIP}_{3-42}$ ) and glucagon-like peptides ( $\text{GLP-1}_{7-36}$  and  $\text{GLP-1}_{9-36}$ ) have been quantified with mass spectrometric approaches (Wolf et al., 2004; Miyachi et al., 2013; Chappell et al., 2014a, 2014b). The quantitation of angiotensin I (AngI), a component of the renin–angiotensin–aldosterone system (RAAS), has previously been reported in order to assess plasma renin activity (PRA) (Bystrom et al. 2011; Chappell et al., 2012).

Urine peptides have been proposed as biomarkers for kidney diseases such as diabetic nephropathy, acute kidney injury, acute allograft rejection, and chronic kidney disease (Rossing et al., 2005; Sigdel et al., 2009; Alkhafaf et al., 2010; Good et al., 2010; Ling et al., 2010; Metzger et al., 2010; Zürbig et al., 2011).

Also for C-reactive protein (CRP), a marker for inflammatory and cardiovascular disease, and prostate-specific antigen (PSA), a marker for prostate cancer, LC-MS methodology has been proposed (Kuhn et al., 2004; Williams and Muddiman, 2009; Yocum et al., 2010), although these biomarkers have  $\text{MW} > 10,000$  and a digestion step was included in the assay. For diagnosis of prostate cancer, a huge need exists to improve the selectivity of the current diagnostic tests using PSA. Therefore, three different peptide biomarkers have been proposed for tissue samples to distinguish indolent from aggressive prostate cancer disease states (Yocum et al., 2010). Multiplexed quantification of protein biomarkers of clinical relevance to cardiac injury including the cardiac troponins was described (Keshishian et al., 2009).

Since 2006 LC-MS/MS quantification of the amyloid peptide biomarkers for Alzheimer's disease (AD) has been introduced as an alternative to existing immunoassay platforms, which showed highly variable results (Oe et al., 2006; Dillen et al., 2011; Lame et al., 2011; Lachno et al., 2012; Choi et al., 2013). These studies have enabled comparison between results obtained from different platforms and these molecules will be further discussed in this chapter.

## 14.2 Challenges and Approaches

### 14.2.1 Multiply Charged Ions: SRM Versus HRMS

Typically, for selective targeted quantitative mass spectrometric methods, SRM is preferred, where one or more peptide-specific fragmentation transitions are monitored. These approaches are routinely applied by the bioanalytical community for the quantitation of

**Table 14.1** List with a selection of some endogenous biomarker peptides (MW < 10,000) with a published quantitative LC-MS assay.

Peptide name	MW	Matrix	Reference
Opioid peptides			
Leucine-enkephalin	555.6	CSF	Sinnaeve et al. (2005)
Methionine-enkephalin	573.7	Pituitary homogenate	Kusmierz et al. (1990)
Breast cancer biomarkers			
Bradykinin	1060.2	Serum	Van den Broek et al. (2010a)
Hyp <sup>3</sup> -bradykinin	1076.2	Serum	
Des-Arg <sup>9</sup> -bradykinin	904.1	Serum	
Fib- $\alpha$ <sub>(605–629)</sub>	2659.8	Serum	
C4a <sub>(1337–1350)</sub>	1626.8	Serum	
ITIH <sub>4</sub> <sub>(666–687)</sub>	2358.6	Serum	Van den Broek et al. (2010b)
Neuropeptides			
Neurotensin	1672.9	Microdialysates	Maes et al. (2014)
Neuromedin B	1132.3	Microdialysates	Maes et al. (2014)
Neuromedin N	746.0	Microdialysates	Maes et al. (2014)
Oxytocin	1006.8	Microdialysates plasma	Mabrouk and Kennedy (2012), Zhang et al. (2011)
Substance P	1347.6	Spinal cord	Beaudry and Vachon (2006)
Endocrine hormone peptides			
Insulin	5808	Plasma	Chambers et al. (2013)
Glucagon	3482.7	Plasma	Howard et al. (2014)
C-peptide	3020	Plasma, serum, urine	Rogatsky et al. (2006a, 2006b), Stoyanov et al. (2011, 2013)
GIP[1–42], GIP[3–42]	4983.6/4759.4	Plasma	Miyachi et al. (2013)
GLP-1[7–36] amide, GLP-1[9–36] amide	3297.7/3089.5	Plasma	Chappell et al. (2014a, 2014b)
Alzheimer's disease markers			
Amyloid peptides: A $\beta$ <sub>1–38</sub> ; A $\beta$ <sub>1–40</sub> ; A $\beta$ <sub>1–42</sub>	4131.6; 4329.9; 4514.1	CSF	Oe et al. (2006), Dillen et al. (2011), Lame et al. (2011), Lachno et al. (2012), Chambers et al. (2013), Choi et al. (2013)
Rheumatoid arthritis markers			
S100-A9 fragment	1900.9	Urine	Stalmach et al. (2014)
Gelsolin fragment	2437.6	Urine	
Uromodulin fragments	1160.3–2040.3	Urine	
Complement C3 fragment	3648.9	Urine	
Fibrinogen fragment	2307.3	Urine	
Angiotensin I	1296.5	Microdialysates	Lanckmans et al. (2007)
Angiotensin II	1046.2		
Vasopressin	1084.2	Microdialysates	Mabrouk and Kennedy (2012)

small molecules. Workflows optimizing the SRM transition to maximize sensitivity and selectivity can be largely automated for small molecules and many vendors offer off-the-shelf software packages. However, for peptide SRM methods, these automated optimization workflows

are less well developed. In silico predictions of the anticipated most abundant fragment ions are available for different types of instruments and different vendors and are applied as well for tryptic fragments in targeted proteomic workflows. Skyline offers open access

software to facilitate targeted quantitative peptide analysis (MacLean et al., 2010a).

In electrospray ionization, proteins and peptides are present as multiply charged ions. The distribution of the charge states is dependent on experimental conditions and on peptide concentration (Hewavitharana et al., 2010; Campbell and Le Blanc, 2011). The majority of literature reports on LC-MS methods for peptides refer to tryptic peptides that are generated in the proteomic workflows to generate the signature peptides of the protein of interest. These tryptic peptides produce mainly doubly and/or triply charged positive precursor ions in electrospray. However, the charge state of endogenous peptides is not nearly as uniform as for tryptic peptides. Some endogenous peptides have no positive charge due to a blocked N-terminus (acetylation or pyroglutamyltation) and the absence of Lys, Arg, or His residues; these peptides cannot be detected in positive ion mode. In contrast, other endogenous peptides have much higher charge states (4+ up to 7+). These variations in peptide size and charge state complicate the analysis of endogenous peptides, especially in comprehensive workflows.

In addition, the mechanisms of fragmentation of peptides are sensitive to small changes in collision energy (CE). Often, limited fragmentation is observed at low CE, while the optimal CE often results in many ladder sequence ions (*b* and *y* ions) (MacLean et al., 2010b), extremely valuable for qualitative identification purposes, but reducing sensitivity in a quantification workflow by dilution of the SRM signal. At higher CE complete fragmentation can result in individual immonium ions that do not discriminate between peptides and therefore are not the preferred choice for development of a selective SRM method.

As we have elaborated earlier (Dillen and Cuyckens, 2013) that for peptide quantification both triple-quadrupole and high-resolution mass spectrometers (TOF, orbitrap) could be considered as options for quantitative LC-MS analysis, triple-quadrupole mass spectrometers are still believed to provide superior sensitivity. However the behavior of peptides in the electrospray ionization process (multiple charges) on the one hand and the fragmentation in the collision cell (poor fragmentation or too much fragmentation) on the other hand often will reduce ion transfer efficiency in the triple-quadrupole SRM acquisition mode. Although ionization impact—due to distribution of the signal over different charge states—is identical in the high-resolution mass spectrometer, selection of the full-scan acquisition mode removes the (inefficient) fragmentation step. Summation of different charge state signals can partially remediate sensitivity losses. Especially in situations with multiple analytes, limitations in scan speed will restrict the number

of possible analytes in triple-quadrupole MRM assays. Alternative approaches have been proposed for peptide analysis such as MRM cubed (MRM<sup>3</sup>) and parallel reaction monitoring (PRM) on the Q-Exactive instruments to improve selectivity and sensitivity (Domon and Aebersold, 2010; Gallien et al., 2012). Nevertheless in practice most absolute—non-comprehensive—quantitative assays still rely on robust triple-quadrupole assays.

#### 14.2.2 Adsorption–Solubility–Stability Aspects

Most peptides have hydrophilic properties and are soluble in aqueous buffers. On the other hand, many peptides show nonspecific binding (adsorption) to containers. Therefore the choice of solubilization solvent (concentration, organic content) and recipients (glass vs. polypropylene but also ratio of volume to surface area) should be carefully considered. Subsequent dilutions for calibration curve preparations also require scrutinized optimization and evaluation to avoid major inconsistencies in results later on. A detailed guidance—based on an extensive investigation—was presented (Maes et al., 2014). Also strategies were proposed to efficiently reduce adsorption in the LC-MS system (autosampler, column). Nevertheless the main conclusion was that a tailored examination is needed for every peptide during method development due to the unique nature of each peptide (Van Eeckhaut and Mangelings, 2015). However, in view of the many multiplexed assays—with analytes of very divergent nature—a best “common generic” strategy needs to be implemented. Understanding and documentation of the accuracy and precision is critical to master variability in the assay.

Both chemical and biochemical (matrix-related) stability needs to be addressed as well. Degradation can occur in the solid state as well as in solution. Different types of chemical degradation have been reported such as deamidation, hydrolysis, oxidation, and many more. In biological matrices peptidases and other enzymes are present and can modify peptides; stabilization of the analytes during sample collection and handling might be required.

#### 14.2.3 Blank Matrix–Internal Standard–Surrogate Analytes

Since biomarkers are endogenous analytes present in the biological matrices studied, biomarker quantification has also been hampered by the lack of analyte-free matrix for preparation of calibration curves and quality control (QC) samples. Alternative or surrogate blank matrices have been proposed ranging from simple buffer solutions to more complex selections such as albumin solutions as substitute for plasma or artificial CSF as substitute for “real” CSF (Dillen et al., 2011).

Some peptide biomarkers are species specific (amino acid composition can differ or biomarker is not present) and in this situation species-surrogate matrix can provide an alternative (e.g., bovine plasma as substitute for human plasma).

However, these alternative matrices can introduce variation in the assay performance since it is very well recognized that ionization of analytes in electrospray is to a large degree influenced by the presence of matrix components. Therefore it is advised and preferred to have as much as possible a similar or identical matrix in calibrator, QC and study samples to control as many aspects as possible of an LC-MS assay (recovery, dilution, adsorption, ionization). In order to compensate for the potential variability, internal standards (IS) are commonly employed in targeted bioanalytical assays. The use of stable isotope-labeled internal standards (SIL-IS) is preferred since these peptides behave (almost) identically to the endogenous non-labeled biomarker under investigation. SIL-IS differs from the endogenous analyte only by a number of mass units. The selection of the nature ( $^{13}\text{C}$ ,  $^2\text{H}$ ,  $^{15}\text{N}$ ) and number of isotopes included will depend on the ease of synthesis and the impact of the isotopic effect of the analyte on the SIL-IS (Gu et al., 2012) as well as on the isotopic effect in chromatography. Co-elution and co-ionization are required for the most optimal correction of the variability. Although structural analog peptides have been evaluated as alternative IS, experience has learned that for some peptides addition or removal/exchange of even one amino acid can bring substantial change in the extraction/chromatography or ionization behavior of the peptides. Also generic small molecules have been proposed as IS to control some of the variability. It is obvious that in case no SIL-IS is available, the utility and quality of the IS needs case by case evaluation.

In the absence of good surrogate matrices, surrogate analytes could also be considered to construct calibration lines. According to this strategy the SIL peptides are now used as standards/calibrators and spiked to the matrix of interest. The endogenous biomarker will be present in the matrix, but the mass shift of the substitute standard compared with the biomarker allows quantification without contribution of the endogenous biomarker. Prerequisite is equivalent LC-MS behavior of the analytes.

With either of the strategies presented standard addition of the analyte(s) to study samples can confirm validity of the approach. Standard addition in general is a valuable tool to monitor accuracy of the assay. However, often standard addition is only considered during method development and/or validation or qualification, mainly driven by sample volume limitations. This approach implies additional workload for real study samples in a routine assay.

#### 14.2.4 Extraction–Sample Pretreatment

Depending on the sensitivity requirements for the peptide biomarker considered, sample pretreatment with the aim to concentrate the analytes of interest might be essential. Even without the specific need for enhanced sensitivity, sample pretreatment to remove matrix components can substantially improve the robustness of the assay. Four commonly used techniques for extraction of peptides from the biological matrices are:

- 1) Protein precipitation (PP)
- 2) Solid-phase extraction (SPE)
- 3) Liquid–liquid extraction (LLE)
- 4) Immunoaffinity-based extractions

Sometimes combinations of these four basic approaches have been applied (e.g., PP with SPE). Depending on the nature and the size of the peptide in combination with the matrix considered, a design for the extraction strategy can be worked out. PP is widely applied for plasma by addition of at least two volumes of acetonitrile. However, inclusion of peptides in the protein precipitate is of particular concern for larger hydrophilic peptides. Alternative precipitants ( $\text{ZnSO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ ) are less MS friendly but can be combined with SPE or LLE. SPE (both hydrophobic or mixed-mode-based extractions) is often applied and can be easily downscaled and automated, even in an online format with the LC-MS instrument.

Immunoaffinity purification offers—in case specific antibodies are available—a nice and selective purification and concentration approach, which, in combination with the mass spec detection, is sometimes referred to as ELISA-MS. It combines the high affinity purification of the immunological approach with the enhanced selectivity provided by LC-MS analysis (no interference from potential degradation or impurity analog analytes). In practice the unavailability of good quality antibodies combined with the complexity of the sample treatment have made researchers reluctant to engage with these lower-throughput approaches. In proteomic workflows enrichment of peptides after digestion with stable isotope standards and capture by anti-peptide antibodies (SISCAPA) is successfully applied to lower the quantification limit by two orders of magnitude (Anderson et al., 2009).

In multiplexed targeted quantification, depletion of the most abundant plasma proteins using immunoaffinity has been achieved as well to capture low abundance proteins and peptides (Keshishian et al., 2007). Shi et al. (2013) described the purification of low levels of peptides in human plasma without immunoaffinity depletion. They combined high-resolution LC separation

with a fractionation with intelligent selection and multiplexing of the targeted peptides for downstream LC-MS analysis in the second dimension.

## 14.3 Application to the Quantification of Alzheimer's Disease Biomarkers

### 14.3.1 Introduction: Amyloid Peptides in CSF as Biomarkers for Alzheimer's Disease

AD is a neurodegenerative disorder characterized by the presence of senile plaques in brain tissue. These brain plaques contain amyloid peptides, which are the products of both normal and abnormal intracellular processing of the amyloid precursor protein (APP) (Hardy and Selkoe, 2002). The amyloid hypothesis suggests that altered A $\beta$  peptide production and/or elimination—especially of the hydrophobic A $\beta$ <sub>1–42</sub>—triggers the formation of the extracellular deposits and the toxicity observed in neurons. Therefore, the levels of these peptides have been evaluated with respect to their use as biomarkers of AD in patients. Significant variations in A $\beta$  peptide levels in plasma and/or in CSF have been reported and hamper interpretation of the data (Blennow and Hampel, 2003; Hansson et al., 2010). Since CSF is in intimate contact with the brain's interstitial fluid, it is considered a good matrix from which biomarkers of central nervous system related diseases can be identified (Blennow and Hampel, 2003; Perret-Liaudet et al., 2012; Lu et al., 2012). With respect to AD and the amyloid peptides, levels of A $\beta$ <sub>1–42</sub> in CSF appear to correlate with aggregation and formation of plaques in the brain (Cirrito et al., 2003; Prince et al., 2004; Fagan et al., 2006), but no clear correlation has been documented between the levels of A $\beta$ <sub>1–42</sub> in plasma and in CSF (Vanderstichele et al., 2000; Mehta et al., 2001).

### 14.3.2 LC-MS/MS Method for Analysis of Amyloid Peptides in CSF in Support of Preclinical Development

Mass spectrometric support by our laboratories for analysis of amyloid peptides (A $\beta$ <sub>1–37</sub>, A $\beta$ <sub>1–38</sub>, A $\beta$ <sub>1–40</sub>, A $\beta$ <sub>1–42</sub>) in dog and human CSF was requested in support of drug development to treat AD patients. Although immunoassays were available, the variability in the results across experiments and the suspected specificity issues justified the development of alternative approaches for the quantitation of these peptides. An LC-MS/MS method provides an additional advantage; new peptides of interest (e.g., A $\beta$ <sub>1–37</sub> or shorter N-terminal fragments) could easily be included in the same assay with limited extra

method development effort. Valuable new peptides can then subsequently be added to existing immunological platforms (alphaLISA, mesoscale). In support of discovery projects, an animal model was used to evaluate the effectiveness of new therapeutic interventions on CSF levels for the four major amyloid peptides in dogs. These experiments were supported by a UHPLC-MS/MS method, which was published earlier (Dillen et al., 2011). Additional explorative evaluations limited the available CSF volume to 100  $\mu$ L for the amyloid assay with LC-MS. Limited sample workup, only partial PP in study samples with acetonitrile, was applied. Key was to add NH<sub>4</sub>OH (both in LC solvents and in the sample prep) to solubilize the amyloid peptides and to mitigate the adsorption to containers.

Correct choice of surrogate matrix for calibration and QC samples was investigated. At this stage, no IS were included in the assay and it is therefore important to control matrix effect. Artificial CSF showed pronounced matrix effect in the LC-MS response. Standard addition was explored but not very practical since limited sample volumes are available for analysis. CSF originating from other species (bovine, goat) was included in the evaluation of substitute matrices. Amyloid peptides do not have identical amino acid sequences across all species (man and dog have the same sequence at the level of the amyloid peptides) and therefore could offer a good alternative substitute matrix if CSF can be obtained easily. Nevertheless cross-species differences in matrix effects were still observed with this assay, and this first assay was considered as a screening assay providing relative concentration levels (comparing levels to a reference, e.g., before start of treatment or vehicle dosed subjects) across experiments using artificial CSF as surrogate matrix. Although no formal method validation was executed, this method had excellent performance (LLOQ for four peptides 0.500 ng/mL or lower).

In this method, quadruply charged parent ions are fragmented in negative ion mode to one predominant ion that results from the loss of one H<sub>2</sub>O molecule. Although this product ion still has an  $m/z > 1000$ , the selectivity of these transitions has been challenged in literature. Other investigators have selected positive ion mode and a transition to a y ion with a mass higher than the mass of the parent molecule due to the loss of a charge (Chambers et al., 2013).

This first assay has been extensively applied in support of discovery programs (Borghys et al., 2012; Borghys et al., 2014). Effects of inhibitors of the gamma secretase enzyme or the  $\beta$ -site amyloid precursor protein-cleaving enzyme (BACE) were evaluated. Dose-dependent decreases in the levels of all measured A $\beta$  peptides were observed with  $\gamma$ -secretase inhibitors, while  $\gamma$ -secretase modulators increased dog CSF levels of the shorter

amyloid peptides ( $\text{A}\beta_{1-38}$ ) in line with the results obtained with a mesoscale assay. Similar findings were obtained with the BACE inhibitors showing dose-dependent decreases in canine CSF levels of the four amyloid peptides.

#### 14.3.3 LC-MS/MS Method for Analysis of Amyloid Peptides in CSF in Support of Clinical Development

A qualified assay for analysis of the amyloid peptides in human CSF was established in our laboratories building on the experiences of the discovery assay. Some of the challenges encountered in the discovery assay were addressed. Positive ion mode was chosen in combination with the selection of a selective  $y$  ion for each peptide. The loss in sensitivity could be compensated for by the SPE sample preparation consuming 500  $\mu\text{L}$  CSF. Matrix effects were neutralized by introduction of SIL-IS for three of the four peptides (not available for  $\text{A}\beta_{1-37}$ ).

Patient CSF samples are aliquoted as 500  $\mu\text{L}$  aliquots in micronic tubes (MP22502) at the clinical site and frozen immediately. This pre-aliquoting prevented the loss of analytes due to adsorption and obviated the need for sample transfers downstream in the analytical lab. SPE extraction was executed with the positive pressure 96-well processor (using 6–12 psi until dry). To the 500  $\mu\text{L}$  aliquot of human CSF or to a 500  $\mu\text{L}$  QC in artificial CSF (a-CSF), 25  $\mu\text{L}$  water/acetonitrile/ammonia (25%) (78/20/2; v/v/v) is added as well as 150  $\mu\text{L}$  of an  $\text{H}_3\text{PO}_4$  solution (4% in water). The combined SIL-IS spiking solution is added (20  $\mu\text{L}$  DMSO 100 ng/mL each of  $\text{A}\beta_{1-38}^{15}\text{N}$ ,  $\text{A}\beta_{1-40}^{15}\text{N}$ ,  $\text{A}\beta_{1-42}^{15}\text{N}$ ) and mixed. For SPE extraction, Oasis MCX 96-well plate 30  $\mu\text{m}$  (10 mg) was used. Following conditioning with 200  $\mu\text{L}$  methanol and equilibration with 200  $\mu\text{L}$  4% of  $\text{H}_3\text{PO}_4$ , two times 200  $\mu\text{L}$  acetonitrile/water/ammonia (25%) (75/15/10; v/v/v) was transferred over the SPE columns. Another rinse with 200  $\mu\text{L}$  water was included before a second conditioning cycle was started. These steps were introduced to remove background originating from the SPE MCX material. After the second conditioning cycle, the prepared sample was loaded onto the 96-well extraction plate. Subsequently 200  $\mu\text{L}$  4%  $\text{H}_3\text{PO}_4$  was added to the SPE wells, followed by 200  $\mu\text{L}$  20% methanol in water and 200  $\mu\text{L}$  20% acetonitrile in water. The amyloid peptides were eluted in two steps: in the first step a 50  $\mu\text{L}$  aliquot of acetonitrile/water/ammonia (25%) (75/15/10; v/v/v) was used; in the second step an additional 25  $\mu\text{L}$  aliquot of the same elution solvent was used. Eluted peptides were collected into micronic tubes and further diluted 1/1 with water. The SPE sample preparation was automated on the Tomtec Quadra 3 SPE. The LC-MS/MS method is described in Tables 14.2 (LC) and 14.3 (MS).

Due to the limited mass range on the Sciex 5500 instrument (up to  $m/z$  1250) the interscan pause time was

**Table 14.2** UHPLC gradient conditions for the amyloid assay.

Time (min)	% B
0	2.5
0.5	2.5
4.5	55
4.6	95
5.0	95
5.1	2.5

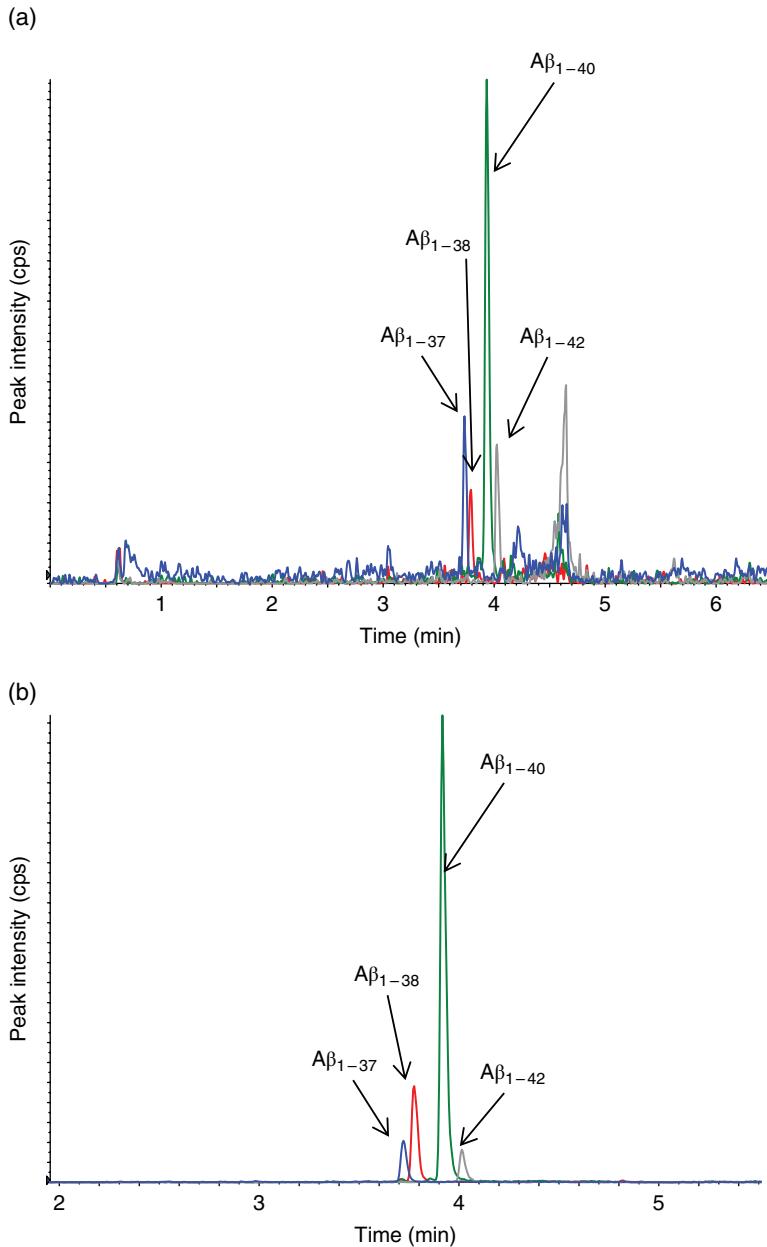
Column: 50 × 2.1 mm, 1.7  $\mu$  ACQUITY UPLC BEH 300 C<sub>18</sub> (Waters), operated at 40°C. Flow rate: 0.25 mL/min, mobile phase A = Milli-Q water, mobile phase B  $\text{CH}_3\text{CN}/\text{CH}_3\text{OH}/\text{TFE}$  (70/25/5; v/v/v) containing 1% ammonia (25%). TFE, trifluoroethanol.

**Table 14.3** MS/MS parameters on Sciex 5500 triple-quadrupole instrument (positive ion mode, IS=5500V, TEM 600°C, CAD gas: medium, curtain gas 25 psi, GS1 and GS2 40 and 50 psi, respectively, entrance potential (EP) 10V, declustering potential (DP) 200V, CXP 4V).

Peptide name	Q1 mass ( $m/z$ )	Q3 mass ( $m/z$ )	Dwell time (ms)	CE (V)
Dummy	1019.6	1000.9	10	28
$\text{A}\beta_{1-37}$	1019.6	1000.9	25	28
$\text{A}\beta_{1-38}$	1033.9	1000.9	25	30
$\text{A}\beta_{1-38}^{15}\text{N}$	1046.1	1012.6	20	30
$\text{A}\beta_{1-40}$	1083.3	1054.0	25	31
$\text{A}\beta_{1-40}^{15}\text{N}$	1096.4	1066.9	20	32
$\text{A}\beta_{1-42}$	1129.5	1078.9	25	36
$\text{A}\beta_{1-42}^{15}\text{N}$	1143.1	1092.0	20	36

automatically increased by the software to 20 ms. In addition, to control the variability of the assay—especially for  $\text{A}\beta_{1-37}$ —the introduction of a dummy transition after the last transition at the highest  $m/z$  (1143.1 > 1092.0 for  $\text{A}\beta_{1-42}$ ) was required to allow cool down of the quadrupoles before the next cycle of scans. API 4000 instruments would offer a broader mass range for SRM transitions, but their scan speed is not sufficient to obtain 10–20 scans over the chromatographic peaks when multiple mass transitions are monitored simultaneously. Figure 14.1 shows the chromatographic trace for the four peptides at LLOQ level; in Figure 14.1b a typical chromatogram of a study sample is presented.

The SPE-LC-MS/MS method was qualified as follows: the calibration range for the different peptides ranged from 0.100 to 20 ng/mL for  $\text{A}\beta_{1-37}$ ,  $\text{A}\beta_{1-38}$ , and  $\text{A}\beta_{1-42}$  and from 0.500 to 100 ng/mL for  $\text{A}\beta_{1-40}$  in artificial CSF. %RE applied for acceptance of the qualification run was 20% (25% at LLOQ).



**Figure 14.1** UHPLC-MS/MS chromatogram of the four amyloid peptides in artificial CSF at LLOQ level (a) and of a typical human CSF sample (b). Method details are described in the text.

For accuracy and precision evaluation, QC samples were prepared in artificial CSF at five levels:

- 1) LLOQ concentration
- 2) LLOQ2 concentration (<3 times the LLOQ)
- 3) Low concentration (three times the LLOQ2)
- 4) Medium (within a factor 2.5 of the median calibration standard)
- 5) High (85-100% of the highest calibration standard)

The results presented in Table 14.4 show precision and accuracies well within acceptance criteria.

Parallelism between the calibration curves in artificial CSF and human CSF was investigated. A limited

calibration curve was prepared in human CSF fortified at the same concentration levels of the amyloid peptides as available in the calibration curve in artificial CSF (endogenous concentration and two concentrations spiked on top of the endogenous level). The Y-axis intercept from the curve prepared in human CSF was compared with the mean calculated endogenous value from the unspiked human CSF samples ( $n=6$ ) as determined using the artificial CSF curve. Linear regression using  $1/x^2$  weighing was used to calculate the intercept of the human CSF curve.

Parallelism was also evaluated by calculating the % accuracy of the fortified human CSF control samples

**Table 14.4** Accuracy and precision results for the amyloid peptides in a-CSF ( $n=6$ ).

		$\text{A}\beta_{1-37}$	$\text{A}\beta_{1-38}$	$\text{A}\beta_{1-40}$	$\text{A}\beta_{1-42}$
$\text{QC}_{\text{LLOQ}1}$	Mean	0.110	0.100	0.511	0.0928
0.100 ng/mL	% CV	5.3	15.3	6.8	6.8
(0.500 ng/mL)	% bias	10.0	0.0	2.2	-7.2
$\text{QC}_{\text{LLOQ}2}$	Mean	0.272	0.252	1.15	0.237
0.250 ng/mL	% CV	8.3	9.8	4.9	10.2
(1.25 ng/mL)	% bias	8.8	0.8	-8.0	-5.2
$\text{QC}_{\text{Low}}$	Mean	0.747	0.742	3.56	0.658
0.750 ng/mL	% CV	7.5	7.5	6.8	2.7
(3.75 ng/mL)	% bias	-0.4	-1.1	-5.1	-12.3
$\text{QC}_{\text{Medium}}$	Mean	2.09	1.98	9.56	1.98
2.00 ng/mL	% CV	4.4	7.4	4.7	8.9
(10.0 ng/mL)	% bias	4.5	-1.0	-4.4	-1.0
$\text{QC}_{\text{High}}$	Mean	21.0	21.1	94.8	18.1
20.0 ng/mL	% CV	4.5	9.7	4.7	3.9
(100 ng/mL)	% bias	5.0	5.5	-5.2	-9.5

Concentration levels for  $\text{A}\beta_{1-40}$  are mentioned within brackets. Acceptance criteria for precision and accuracy were  $\leq 20\%$  CV and  $\leq 20\%$  ( $\leq 25\%$  at LLOQ) bias, respectively.

**Table 14.5a** Standard addition (1 and 10 ng/mL except for  $\text{A}\beta_{1-40}$  with additions of 5 and 50 ng/mL) of amyloid peptides to human CSF ( $n=6$ )—analyzed with a calibration curve in artificial CSF.

		$\text{A}\beta_{1-37}$	$\text{A}\beta_{1-38}$	$\text{A}\beta_{1-40}$	$\text{A}\beta_{1-42}$
hCSF	Mean	1.16	4.99	17.1	1.00
Endogenous	% CV	4.5	2.6	4.4	10.8
	% bias	na	na	na	na
hCSF	Mean	2.16	5.99	22.1	2.00
e + 1.00 ng/mL	% CV	6.0	2.7	2.1	3.7
(e + 5.00 ng/mL)	% bias	9.7	5.2	1.8	1.5
hCSF	Mean	11.2	15.0	67.1	11.0
e + 10.0 ng/mL	% CV	2.1	1.2	4.1	2.9
(e + 50.0 ng/mL)	% bias	17.9	12.7	3.1	4.5

na, not applicable; e, endogenous.

using the artificial CSF curve (Table 14.5a). All accuracies were within acceptance criteria. Differences based on comparison of PARs obtained from endogenous level and the PAR obtained as the result of the intercept of the fortified hCSF curve to Y-axis are well within acceptance (<20%) (Table 14.5b).

A standard stability program was executed. Freeze/thaw stability (four cycles), bench top stability for 4 hours at room temperature and 2 h at 37°C were proven for the four amyloid peptides. Long-term stability for 267 days

**Table 14.5b** Parallelism based on % difference of peak area ratio (PAR) in human CSF compared with PAR obtained from the intercept of the human CSF curve.

	$\text{A}\beta_{1-37}$	$\text{A}\beta_{1-38}$	$\text{A}\beta_{1-40}$	$\text{A}\beta_{1-42}$
Mean PAR of endogenous level	0.121	0.292	1.28	0.196
PAR from intercept hCSF curve	0.124	0.297	1.26	0.171
% difference	-2.8	-1.8	0.9	13.5

(−20°C) was confirmed for  $\text{A}\beta_{1-37}$ ,  $\text{A}\beta_{1-40}$ , and  $\text{A}\beta_{1-42}$  but not for  $\text{A}\beta_{1-38}$ . Recovery was around 70% for all analytes. Absolute matrix effects were most pronounced for  $\text{A}\beta_{1-42}$  (MF 0.56 in hCSF and 0.67 in aCSF), but IS normalized matrix factor was close to 1.

#### 14.3.4 Comparison of Immunoassay and UHPLC-MS/MS: Are the Results Comparable?

Different assays are used to measure  $\text{A}\beta$  peptides in CSF (and other matrices). Immunoassays (Bjerke et al., 2010; Head et al., 2010), mass spectrometric assays (UHPLC-MS/MS) in combination with a variety of sample preparation methods (SPE extraction, immunoprecipitation) have been described (Oe et al., 2006; Portelius et al., 2006; Gelfanova et al., 2007; Chambers et al., 2010; Dillen et al., 2011). Critical aspects mentioned in many publications are related to certified reference materials, and the sampling and processing procedures applied. Therefore comparing absolute concentration levels across different laboratories is challenging and often results in discrepancies. The variability in CSF biomarker measurements for AD is considered a major obstacle for the establishment of decision points in diagnosis and therapeutic intervention. Several initiatives have focused on presenting reference measurement procedures and on the availability of certified reference materials (Mattsson et al., 2012).

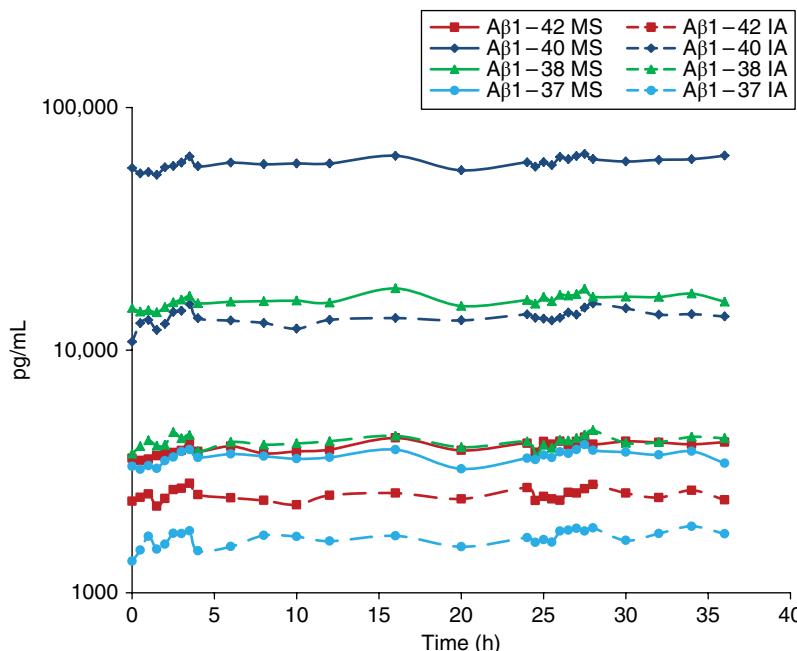
Since in our laboratories both immunoassay and an LC-MS/MS method were developed, both technologies were applied in a number of dedicated studies. Dog CSF samples were analyzed with two assays: the mesoscale immunoassay and the above described discovery UHPLC-MS/MS assay. In early development programs, baseline concentration levels were monitored in dogs. CSF was sampled from both the lateral ventricle and the cisterna magna. Table 14.6 presents the data. Two observations are evident: baseline  $\text{A}\beta$  concentrations are in general about two times higher in the cisterna magna compared with the lateral ventricle, and the UHPLC-MS/MS assay results in higher concentration levels than the immunoassay, the ratio apparently being peptide dependent. For further information and discussion, we refer to the

**Table 14.6** Absolute concentrations (ng/mL, mean  $\pm$  SD) of amyloid peptides in dog CSF analyzed with UHPLC-MS/MS and mesoscale and the ratios of concentrations determined by the two assays.

	Lateral ventricle			Cisterna magna		
	UHPLC-MS/MS	Mesoscale	Ratio	UHPLC-MS/MS	Mesoscale	Ratio
A $\beta$ <sub>1-37</sub>	0.495 $\pm$ 0.223	0.282 $\pm$ 0.099	1.8	0.803 $\pm$ 0.224	0.497 $\pm$ 0.135	1.7
A $\beta$ <sub>1-38</sub>	2.49 $\pm$ 0.767	0.924 $\pm$ 0.341	2.8	4.31 $\pm$ 1.19	1.820 $\pm$ 0.741	2.6
A $\beta$ <sub>1-40</sub>	6.82 $\pm$ 2.44	2.68 $\pm$ 0.811	2.6	13.65 $\pm$ 2.84	3.93 $\pm$ 1.24	3.7
A $\beta$ <sub>1-42</sub>	1.93 $\pm$ 0.732	0.550 $\pm$ 0.168	3.7	4.25 $\pm$ 0.960	0.991 $\pm$ 0.321	4.6

Adapted from Borghys et al. (2014).

Baseline samples,  $n = 99$  for lateral ventricle and  $n = 48$  for cisterna magna.



**Figure 14.2** Mean concentration time profiles of the amyloid peptides in CSF of human volunteers ( $n=6$ ) over a 36 h period as analyzed with two analytical methods. IA, mesoscale immunoassay; MS, UHPLC-MS/MS method.

published paper (Borghys et al., 2014). Also details of the mesoscale assay are described in that manuscript.

A similar observation was made in an explorative clinical study. This clinical study was executed to evaluate the effect of CSF sampling schedule on the A $\beta$  levels in elderly volunteers. Preliminary results had demonstrated that intensive CSF sampling frequencies may impact the A $\beta$  concentration time profiles. Therefore it was proposed to monitor amyloid peptide levels in CSF of volunteers ( $n=6$ ) over 36 h at a reduced CSF sampling frequency (28 time points). This frequency was expected to provide sufficient sampling points to monitor changes in A $\beta$  time profiles induced by drug effects.

The CSF samples from this study were analyzed with an immunoassay and the SPE-UHPLC-MS/MS assay. Figure 14.2 shows the profiles for all peptides analyzed in CSF from volunteers over a 36 hour time period. It is obvious that also in human CSF samples the difference

**Table 14.7** Ratio and % CV of amyloid beta levels in CSF of volunteers obtained with two assays: UHPLC-MS/MS versus Mesoscale Elisa.

	A $\beta$ <sub>1-37</sub>	A $\beta$ <sub>1-38</sub>	A $\beta$ <sub>1-40</sub>	A $\beta$ <sub>1-42</sub>
Ratio MS/IA	2.17	3.84	4.35	1.57
% CV	5.75	5.33	6.25	6.27

between the immunoassay results and the UHPLC-MS/MS data is present. Circadian fluctuations were not observed. Table 14.7 gives the calculated ratio between the CSF concentrations obtained with the UHPLC-MS/MS assay and the immunoassay. The data are indicative of an intrinsic but consistent difference between the two assays. Compared with the ratios obtained in the pre-clinical study, the ratio for A $\beta$ <sub>1-42</sub> seems to deviate (ratio

of MS/IA for preclinical dog CSF samples was 4.2 vs. 1.6 in the clinical CSF samples). All data obtained clearly show an absolute difference in A<sub>β</sub> levels in study samples when analyzed with the two assays; however the profiles and changes over time are comparable and independent of the assay used.

Further experiments were designed to understand the root cause of these differences. All data presented above were generated in two independent labs. To minimize variability, it was decided to centralize the immunoassay and UHPLC-MS/MS assay in one lab by the same analyst and starting from the same stock solutions of amyloid peptides. Twenty pools of dog CSF samples were collected for this investigation. One hypothesis for the discrepancy between the concentration levels obtained with the two methods relates to the potential aggregation and protein binding of these peptides. Sample preparation in the MS assay and/or the LC or MS conditions is believed to reduce aggregation/protein binding, explaining the higher levels. If this hypothesis is correct, addition of a chaotropic agent (guanidinium chloride) would not change levels in the UHPLC-MS/MS assay but could increase the mesoscale readout. A similar approach was examined by Slemmon et al., in 2012. These investigators combined chaotropic treatment with an LC separation and fractionation, followed by an immunoassay of the different LC fractions and reported higher levels after this treatment.

A number of different approaches were compared and evaluated (in all MS-analyses, IS were included) for analysis of the pooled dog CSF samples (see Figure 14.3):

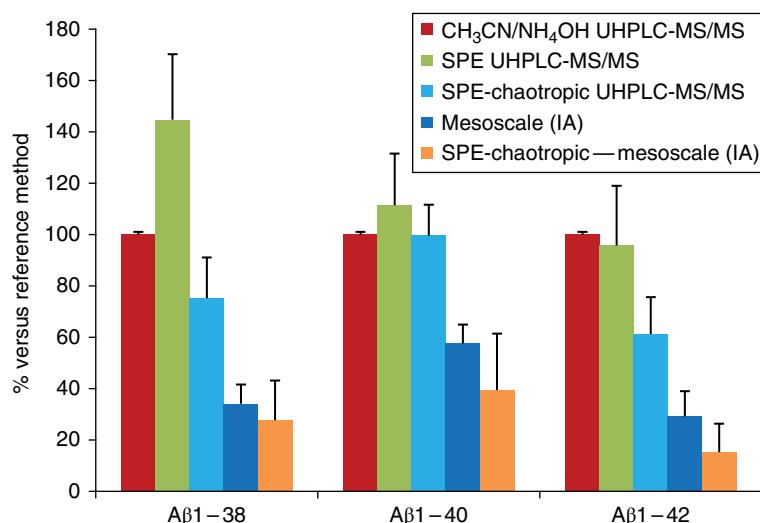
- 1) NH<sub>4</sub>OH/acetonitrile sample preparation—negative electrospray-UHPLC-MS/MS
- 2) SPE sample preparation—positive electrospray-UHPLC-MS/MS

- 3) SPE sample preparation (including addition of chaotropic agent, 7 M guanidinium chloride)—negative electrospray-UHPLC-MS/MS
- 4) Mesoscale without sample preparation
- 5) SPE sample preparation (including addition of chaotropic agent)—mesoscale

The results comparing SPE extracted samples in positive and negative electrospray (data not included in Figure 14.3) showed average ratios of 1.02, 1.08, 1.16, and 0.84 for A<sub>β1-37</sub>, A<sub>β1-38</sub>, A<sub>β1-40</sub>, and A<sub>β1-42</sub>, respectively, providing evidence that results are independent of the ionization mode chosen. Since no SIL-IS was available for A<sub>β1-37</sub> and concentration levels are very low, this peptide was not further included in this evaluation. The summary of the normalized results for the five approaches is presented in Figure 14.3. These data confirm the earlier reported differences between the assays. However, adding a chaotropic agent did not result in increased peptide levels with the immunoassay; instead, even lower levels were obtained. Calibrators and QC samples were also processed in the presence of the chaotropic agents and complied with the acceptance criteria. These experiments did not provide evidence that aggregation/protein binding can explain the observed differences.

In general, for protein and peptide quantification—but in contrast to the findings for amyloid peptides—when immunoassay results are compared with LC-MS results, higher concentration levels are more often measured with immunoassay and can often be explained by (a lack of) selectivity of the antibody (metabolites also recognized by the antibody). A potential oxidation or other minor modification to the peptides in incurred samples changes the MW of the peptides, and this entity will subsequently escape detection in an LC-MS/MS assay, while in immunoassay some modifications will go unnoticed,

**Figure 14.3** Amyloid peptide concentrations in dog CSF samples ( $n=20$ ) obtained with different assays. Levels are expressed relative to first described preclinical assay (= reference method using CH<sub>3</sub>CN/NH<sub>4</sub>OH pretreatment with UHPLC-MS/MS).



and as a consequence, overestimation of the concentration levels can happen. However, in stress stability testing, no impact on stability of the amyloid peptides was observed when an LC-MS assay was used. Also in literature (Pannee et al., 2013), higher concentrations for ELISA-measured amyloid levels in human CSF were reported. A twofold difference in CSF concentration levels was reported for  $\text{A}\beta_{1-42}$ . These investigators also studied the influence of guanidinium chloride but did not observe any difference. More important is that the diagnostic accuracy of both methods (LC-MS/MS and ELISA) was comparable. Until now the observed difference in absolute concentration levels between mesoscale ELISA and the LC-MS/MS remains unexplained.

## 14.4 Conclusion

Although LC-MS/MS methods are sufficiently robust and sensitive to quantify amyloid peptides in CSF, the throughput is still lower compared to immunoassays. Many immunoassays are currently available and have

improved reproducibility and robustness. Therefore in large clinical programs the preferred choice is often the immunoassay. LC-MS/MS assays have been proposed as reference methodology to support or validate the immunoassay. It is critical to have orthogonal technology available to control the validity of an assay over time. Moreover, in early development programs with limited number of samples in exploratory experiments, an LC-MS/MS assay can offer better efficiency (no critical reagents need to be prepared) and flexibility to include more analytes of interest in the assay. An LC-MS/MS assay also offers the potential to screen for the presence of unexpected metabolites or biomarkers.

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## 15

### Targeted Protein Biomarker Quantitation by LC-MS

*Yongle Pang<sup>1</sup>, Chuan Shi<sup>2</sup>, and Wenying Jian<sup>3</sup>*

<sup>1</sup> Department of Chemistry, Michigan State University, East Lansing, MI, USA

<sup>2</sup> Case Western Reserve University, Cleveland, OH, USA

<sup>3</sup> Bioanalytical & Pharmacokinetics, Janssen Research & Development, LLC, Spring House, PA, USA

#### 15.1 Introduction

According to the definition from National Institutes of Health, biological marker (biomarker) is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group, 2001). Biomarkers can be of different forms, such as small molecules, peptides, proteins, lipids, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA), and can be used for detecting disease, forecasting the natural progress of disease, and predicting the response to a treatment (Rifai et al., 2006; Winter et al., 2013; Ansari et al., 2014). Though early work had been strongly focused on nucleic acid-based biomarkers such as DNA, single nucleotide polymorphism (SNP), and messenger RNA (mRNA) expression profiles, recent research has been shifted toward utility of protein biomarkers, which could offer a significantly greater degree of differentiating information. In the past decades, protein biomarker research has gained remarkable interest and progress in the research of different diseases. In the area of oncology, the Food and Drug Administration (FDA) has approved ~20 protein biomarkers for use in clinical practice, as listed in Table 15.1 (Fuzery et al., 2013). There are also numerous examples of emerging protein biomarkers in other disease areas. For cardiovascular diseases, panels of proteins such as cytokines, apolipoproteins, coagulation factors, and so on have been identified as emerging biomarkers (Vasan, 2006; Yayan, 2013). For systemic autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and systemic sclerosis, protein biomarkers such as rheumatoid factor, C-reactive protein (CRP), antinuclear antibody, anti-dsDNA antibody, and antiphospholipid antibody have been investigated

though evidence has shown that “one protein–one disease” is not likely due to the complex systemic changes in these diseases (Liu et al., 2005). In the neurological area, novel protein biomarkers for common diseases such as Parkinson’s disease, Alzheimer’s disease, and schizophrenia are being identified, and further validation of these biomarkers in large-scale clinical studies is necessary to assess their value in clinical diagnosis (Falkai and Moller, 2012; Kiddle et al., 2015; Halbgewauer et al., 2016). For infectious diseases, a large number of proteins such as CRP, soluble triggering receptor expressed on myeloid cells 1 (sTREM-1), pancreatic stone protein/regenerating protein, interleukin 6 (IL-6), IL-8, IL-27, and soluble urokinase-type plasminogen activator receptor (suPAR), among others, have been studied as potential biomarkers to facilitate diagnosis and prognosis in bacterial sepsis (Mohan and Harikrishna, 2015).

The pipeline of biomarker development consists of a series of stages, generally categorized into discovery, qualification, verification, validation, and clinical evaluation (Rifai et al., 2006). Different assay platforms are used for protein biomarkers for different phases. In the discovery stage, mass spectrometry (MS)-based nontargeted approach (shotgun proteomics) is used for relative quantitation of thousands of proteins in a small number of samples to reveal the differentially expressed proteins as potential candidates for further investigation. After the discovery stage, multiple reaction monitoring (MRM) in tandem mass spectrometry (MS/MS) has often been used for targeted analysis of selected proteins for verification purposes. The advances in MS technology have enabled acquisition of up to hundreds of MRM transition in a single run for monitoring of panels of proteins. In addition, a variety of isotopic labeling techniques have been utilized for relative quantitation, including isobaric tagging (e.g., iTRAQ or TMT tagging)

**Table 15.1** List of FDA-approved protein tumor markers currently used in clinical practice.<sup>a</sup>

Biomarker	Clinical use	Cancer type	Specimen	Methodology	Submission type	Year first approved or cleared	Device class	Product code
Pro2PSA	Discriminating cancer from benign disease	Prostate	Serum	Immunoassay	PMA	2012	3	OYA
ROMA (HE4 + CA-125)	Prediction of malignancy	Ovarian	Serum	Immunoassay	510(k)	2011	2	ONX
OVA1 (multiple proteins)	Prediction of malignancy	Ovarian	Serum	Immunoassay	510(k)	2009	2	ONX
HE4	Monitoring recurrence or progression of disease	Ovarian	Serum	Immunoassay	510(k)	2008	2	OIU
Fibrin/fibrinogen degradation product (DR-70)	Monitoring progression of disease	Colorectal	Serum	Immunoassay	510(k)	2008	2	NTY
AFP-L3%	Risk assessment for development of disease	Hepatocellular	Serum	HPLC, microfluidic capillary electrophoresis	510(k)	2005	2	NSF
Circulating tumor cells (EpCAM, CD45, cytokeratins 8, 18+, 19+)	Prediction of cancer progression and survival	Breast	Whole blood	Immunomagnetic capture/immune-fluorescence	510(k)	2005	2	NQI
p63 protein	Aid in differential diagnosis	Prostate	FFPE tissue	Immunohistochemistry	510(k)	2005	1	NTR
c-Kit	Detection of tumors, aid in selection of patients	Gastrointestinal stromal tumors	FFPE tissue	Immunohistochemistry	PMA	2004	3	NKF
CA19-9	Monitoring disease status	Pancreatic	Serum, plasma	Immunoassay	510(k)	2002	2	NIG
Estrogen receptor (ER)	Prognosis, response to therapy	Breast	FFPE tissue	Immunohistochemistry	510(k)	1999	2	MYA
Progesterone receptor (PR)	Prognosis, response to therapy	Breast	FFPE tissue	Immunohistochemistry	510(k)	1999	2	MXZ
HER-2/neu	Assessment for therapy	Breast	FFPE tissue	Immunohistochemistry	PMA	1998	3	MVC
CA-125	Monitoring disease progression, response to therapy	Ovarian	Serum, plasma	Immunoassay	510(k)	1997	2	LTK
CA15-3	Monitoring disease response to therapy	Breast	Serum, plasma	Immunoassay	510(k)	1997	2	MOI
CA27.29	Monitoring disease response to therapy	Breast	Serum	Immunoassay	510(k)	1997	2	MOI
Free PSA	Discriminating cancer from benign disease	Prostate	Serum	Immunoassay	PMA	1997	3	MTG
Thyroglobulin	Aid in monitoring	Thyroid	Serum, plasma	Immunoassay	510(k)	1997	2	MSW

Nuclear mitotic apparatus protein (NuMA, NMP22)	Diagnosis and monitoring of disease (professional and home use)	Bladder	Urine	Lateral flow immunoassay	PMA	1996	3	NAH
Alpha-fetoprotein (AFP) <sup>b</sup>	Management of cancer	Testicular	Serum, plasma, amniotic fluid <sup>b</sup>	Immunoassay	PMA	1992	3	LOK
Total PSA	Prostate cancer diagnosis and monitoring	Prostate	Serum	Immunoassay	PMA	1986	2	LTJ, MTF
Carcino-embryonic antigen	Aid in management and prognosis	Not specified	Serum, plasma	Immunoassay	510(k)	1985	2	DHX
Human hemoglobin (fecal occult blood)	Detection of fecal occult blood (home use)	Colorectal	Feces	Lateral flow immunoassay	510(k)–CLIA waived	1976	2	KHE

Reprinted with permission Fuzery et al. (2013).

<sup>a</sup> While hCG is commonly used as a tumor marker, it has not been cleared/approved for this application by the FDA.

<sup>b</sup> AFP is a Class III analyte because of its noncancer intended use (aid in prenatal diagnosis of birth defects).

or nonisobaric tagging (e.g., mTraq or acetylation), and label-free quantitation techniques such as spectral counting (Ross et al., 2004; Asara et al., 2008; Chahrour et al., 2015). However, MS technologies used in the early stage of biomarker discovery and verification are generally not transferrable to the final stage of clinical practice due to their high complexity, relative low throughout, and requirement of high operational skills. Ligand binding assay (LBA) such as enzyme-linked immunosorbent assay (ELISA) is still the gold standard for protein biomarker quantitation. LBA methods are low cost, simple with high throughput, and highly sensitive, being able to detect pictogram per milliliter level protein in a biological matrix. As shown in Table 15.1, all of the FDA-approved cancer protein biomarkers in serum/plasma are based on LBA, except for AFP-L3, which utilizes HPLC and capillary electrophoresis.

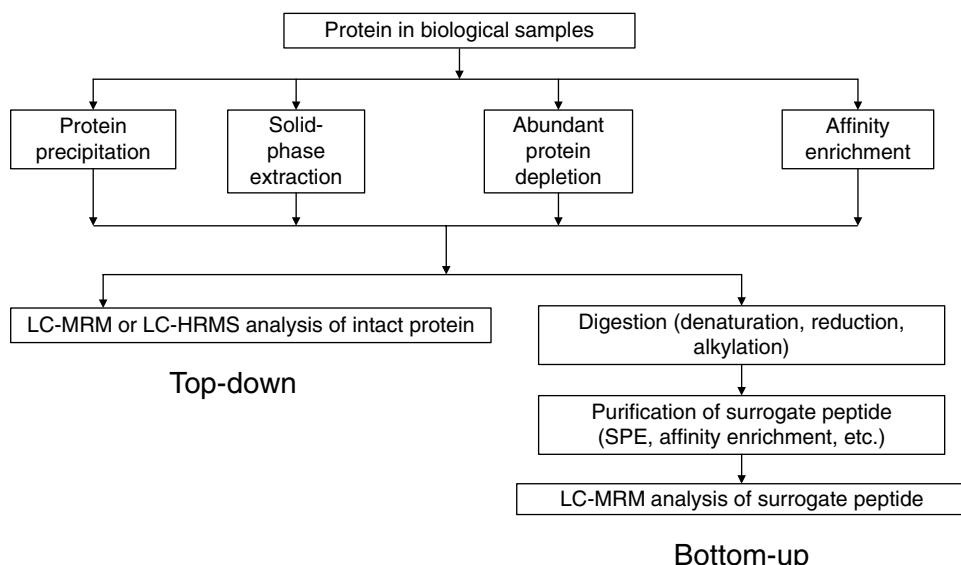
LBA does have its limitations. It requires antibodies with high specificities for the targeted protein. Developing LBA could be very expensive (\$2–4 million for an FDA-approvable assay) and time consuming (1–1.5 years) (Carr and Anderson, 2008). Though having high throughput capabilities, LBA could usually quantify only one protein or a few in one assay, which is not effective when many biomarkers of interest exist in one sample. LBA also suffers from cross-reactivity, lacking of concordance among different platforms, and endogenous nonspecific/multispecific immunoglobulins binding to reagent antibodies (Hoofnagle and Wener, 2009). Another important limitation for LBA is hook effect, which refers to the phenomenon where a sample with a high analyte concentration gives a signal

much lower than a theoretically expected value, due to saturation of binding sites on the capture or detection antibody. Moreover, LBA requires meticulous investigation of parallelism to confirm that the sample dilution-response curve is parallel to the standard concentration-response curve, adding complexity of operation and slowing the throughput. In addition, measurement of protein of interest from different matrices of different species is quite challenging for a single LBA method due to significant impact of the matrix on the assay performance (An et al., 2014). In contrast, liquid chromatography-mass spectrometry (LC-MS) based assays have unique advantages, such as high specificity, fast method development, broad dynamic range, and multiplex capability (Table 15.2). Research has also shown that MRM assays can be highly reproducible within and across laboratories and instrument platforms (Addona et al., 2009). The above advantages have made LC-MS an important complementary tool to LBAs for targeted biomarker quantitation, particularly for verification/validation of protein biomarker candidates and in the application of novel biomarkers during preclinical studies and clinical trials of drug development.

The application of MS-based targeted protein biomarker quantitation can be done by two ways, “bottom-up” and “top-down,” the former of which is the current mainstream approach while the latter has emerged a promising direction. Figure 15.1 shows a general workflow for both “bottom-up” and “top-down” methods. For “bottom-up” approach, protein quantitation is conducted by measuring surrogate peptides (also named

**Table 15.2** Comparison of LBA and LC-MS-based assays on targeted protein biomarkers quantitation.

	LC-MS	LBA
Antibody requirement	Usually not required, or require one antibody for immunoaffinity enrichment	Two antibodies required, highly rely on critical reagent
Development time	Short, days-weeks	Long, months
Specificity	High	May be low due to cross-reactivity
Dynamic range	$10^3$ – $10^5$	$10^2$ , may be subject to hook effect
Variability and interference	Low; less susceptible to interference	High; may be susceptible to interference from endogenous target and anti-drug antibody
Multiplex capability	High	Low
Assay transferrable	Relatively easy to transfer between matrix and species	Matrix and species selective
Structure information (biotransformation, posttranslational modification, isoforms)	Can be obtained	Usually not obtainable
Sensitivity	ng/mL– $\mu$ g/mL	pg/mL
Throughput	Low to moderate	High



**Figure 15.1** General workflow of “Bottom-up” and “Top-down” approach for protein biomarker quantitation.

as signature peptides) generated from enzymatic cleavage of the target protein, and in “top-down” method the intact protein is directly analyzed by the mass spectrometer. While “bottom-up” is still the most popular MS-based method for protein biomarker quantitation, development of high-resolution MS (HRMS) allows “top-down” experiments to run in full scan mode with high accuracy for analysis of proteins at intact level. It gives important structure information such as protein PTMs as well as isoforms. Conventionally, this approach is usually applied to analytes with relatively small molecular weight (<10 kDa) due to the limitation in mass range and resolution of the instrument. The sensitivity could also be restrained as the MS signal is diluted by multiple charge states in electrospray ionization (ESI). Recent advances in sample preparation and MS have provided new opportunities for the “top-down” approach to thrive (van den Broek and van Dongen, 2015; Jian et al., 2016). It has shown great promise as a complementary platform to the “bottom-up” approach for protein biomarker quantitation. More detailed description of “bottom-up” and “top-down” approaches for targeted protein biomarker quantitation and discussion about their recent development will be covered in the following sections.

There are many challenges for protein quantitation using LC-MS-based methods. One of the major problems is that the biological fluid is quite complex. Plasma is the most widely used biofluid for bioanalysis, since it is relatively easy to be obtained, and is a reproducible sample. However, the dynamic range of protein abundance in plasma is from millimolar concentration of albumin to femtomolar of tumor necrosis factor, which crosses 12 orders of magnitude. High abundance of endogenous

interferences (mg/mL) makes quantification of the low abundance protein biomarkers (cancer biomarkers normally present in ng/mL) quite challenging. Major concerns include ion suppression (suppress ionization of the protein biomarker or its surrogate peptide) and transition interference (identical precursor/product ion pairs from interference). Posttranslational modification (PTM) is another challenge for protein quantification. Any PTM on the surrogate peptides will result in signal losing in MRM-based detection, since the mass of precursor ion will be different. Approaches have been developed to overcome these problems, such as adding stable isotope labeled (SIL) internal standard (IS), and *in silico* detecting the interferences and unreliable transitions using software programs. Other approaches aiming at increasing the sensitivity and specificity of LC-MS-based assays include depletion of the abundant plasma proteins, enrichment by immunocapture of the target protein, or immunocapture of the proteolytic peptides from the protein. High pg/mL to low ng/mL level of protein biomarker can be quantified using a combination of different sample treatment methods.

## 15.2 Sample Preparation for Targeted Protein Biomarker Quantitation

Quantitation of protein in biological samples using LC-MS method normally requires upfront cleanup or enrichment to reduce the complexity of the matrices. Otherwise, large amount of endogenous proteins in the

biological matrix may suppress ionization of the low amount targeted protein biomarker, causing poor sensitivity and high variability. This problem must be addressed when setting up a quantitation method by applying appropriate sample cleanup/enrichment procedures. Different sample preparation techniques can be chosen based on the nature of the analyte (molecule weight, abundance, physiochemical properties, sensitivity requirement) and that of the matrix. Sample preparation is usually the bottleneck in the entire workflow and is time- and labor-consuming. Common sample cleanup techniques include, but is not limited to, protein precipitation (PPT), solid phase extraction (SPE), depletion of high-abundant protein, and affinity capture. Details of these techniques will be introduced in the following sections.

### 15.2.1 Protein Precipitation

PPT with miscible organic solvent is widely used to remove abundant proteins in a biological matrix. This method is usually conducted by addition of organic solvents (typically methanol or acetonitrile) in at least 2:1 volume ratio of solvents to plasma, followed by removing the resulting precipitates by centrifugation. Organic solvent decreases the dielectric constant of the plasma solution, which increases attraction between charged polypeptide chains of proteins. It also displaces the well-arranged water molecules around the hydrophobic regions on the protein surface, which minimized the hydrophobic interactions between proteins. When electrostatic interactions become predominant, protein aggregation will happen. Using this method, large endogenous plasma proteins can be effectively depleted (>90%) while small peptides and proteins retain in the solvent (Polson et al., 2003). Alternative reagents for precipitation of plasma proteins include trifluoroacetic acid and zinc sulfate, but they may introduce interferences with LC-MS, and must be removed prior injection. PPT is a cost-effective approach that normally provides sufficient sample cleanup for LC-MS analysis and requires minimum method development. However, it is not a selective method, and coprecipitation of protein analytes can result in significant loss of targeted analytes. More importantly, it cannot be applied to large size protein analytes which will be precipitated. Overall, PPT has limited application for quantitation of protein biomarkers, which usually exist at low abundance and requires highly sensitive and selective methods.

### 15.2.2 Solid Phase Extraction

Another approach to extract proteins from biological matrix is SPE. It is a more selective method than PPT and may require more method development based on

the chemistry of analytes. Ion exchange (IEX) SPE isolates analytes based on differences on electrostatic interaction between charged protein functional groups and oppositely charged stationary phase. It is usually conducted by adjusting the pH of mobile phase to control retention or elution of the analytes. Reverse phase (RP) SPE utilizes hydrophobic interaction to separate analytes, and is considered as a less selective retention mechanism. It has been extensively used for eliminating salts which are introduced by upstream applications, including protein denaturation and disruption of protein-protein binding. In one example, RP SPE was used in our laboratory to extract different glycosylation forms of apolipoprotein C3 (ApoC3 molecular weight around 8.8–9.7 kDa) from human plasma as a potential biomarkers of diabetes (Jian et al., 2013). RP SPE coupled with IEX SPE is designed to maximize the selectivity, but may result in potential sample loss (Yang et al., 2007). Another layer of selectivity can be achieved by choosing SPE sorbent with appropriate pore size, since large endogenous proteins can be removed by size exclusion. A common example is that most proteins larger than 20 kDa cannot enter 40–80 Å sized pores, and can be eliminated during wash steps. SPE is often used to extract proteins of relatively high abundance, or in combination with other sample preparation techniques for further cleaning of the samples, such as purification of surrogate peptides from the proteolytic digestion mixture.

### 15.2.3 Abundant Protein Depletion

Albumin and immunoglobulin together make up 60–80% of total serum protein, and the 22 most abundant proteins represent 99% of total protein content in serum. They significantly complicate the analysis of low abundant proteins (Bjorhall et al., 2005). When a suitable enrichment method for protein or peptide analyte is not available, removal of abundant proteins by immunoaffinity or chemical-affinity can be a simple way to reduce the complexity of matrix, increase the dynamic range, and improve the quantitation sensitivity (Martosella et al., 2005). Depletion kits are commercially available for elimination of albumin, IgG, and other high abundant proteins. Evaluation and comparison of different depletion kits has been reported. The signal to noise ratio of target analytes could increase up to seven fold by removing up to 85% of total protein content in serum (Bjorhall et al., 2005; Anderson and Hunter, 2006; Polaskova et al., 2010; Holewinski et al., 2013). However, depletion process has the potential to remove proteins of interest which are noncovalently bonded to the immunoglobulins or albumin, and coprecipitation of other proteins such as cytokines has also been reported. Therefore, assessment of potential loss of target protein is essential

(Granger et al., 2005). In one example of quantifying prostate-specific antigen (PSA) in serum, a tumor marker for early detection and monitoring of prostate cancer, a certain type of albumin depletion column was shown to give very poor recovery (5%) due to nonspecific binding of the analyte to the resin packed in the column (Fortin et al., 2009). Moreover, the variation in depletion efficiency may increase the chances of pre-analytical biases, and QC samples in authentic biological matrix are necessary to confirm the reliability and reproducibility of depletion process (Seam et al., 2007). Recently, Liu et al. reported a method to remove albumin from serum/plasma using a combination of organic solvent and trichloroacetic acid (Liu et al., 2014). When plasma sample was subject to PPT with 1% trichloroacetic acid in isopropanol, 95% of the total albumin in plasma can be removed while almost 100% of protein analyte was retained in the precipitate, which was further subjected to digestion and LC-MS analysis, a process coined as pellet digestion (Ouyang et al., 2012).

#### 15.2.4 Affinity Enrichment

Affinity enrichment allows specific capture of protein analytes through reversible interaction with immobilized antibodies or ligands. The most selective affinity enrichment is immunocapture, which is normally achieved by using antibody against target protein or peptide. Immunocapture in combination with LC-MS (often referred to as “hybrid-LC-MS”) can give an excellent performance on sample cleanup and protein enrichment for sensitive and reliable quantitation assays, and has been shown to quantify protein biomarkers at the ng/mL range in serum (Whiteaker et al., 2007). In one example of quantifying matrix metalloproteinase 9 (MMP9) in mouse serum, MMP9 antibody cross-linked to protein G magnetic beads was used to capture the targeted analyte from mouse serum. In combination with nanoflow LC and MRM, low ng/mL of MMP9 could be quantified from processed samples corresponding to 5 µL of serum injected to the LC-MS system (Ocana and Neubert, 2010). When specific antibodies against target protein and peptide are not available, a more generic antibody or binding agent can be used to capture the common region of the analytes. For example, protein A and protein G, as well as anti-Fc antibody, are widely used for capturing therapeutic mAbs via reversible binding with Fc region of IgG (Lu et al., 2009). Lectin affinity chromatography was reported to effectively capture glycoproteins. Plavina et al. combined abundant protein depletion with lectin affinity capture to push the limit of detection for glycosylated proteins to low ng/mL (Plavina et al., 2007). Different from LBA, where the assay performance highly

relies on specificity of the antibody, MS detection provides an additional layer of selectivity and the quality of antibody becomes less critical for an LC-MS-based assay. Very often, antibodies that are failed to be developed into reagent for LBA can be used for immunoaffinity capture in the hybrid LC-MS assay.

### 15.3 “Bottom-Up” Approach for Targeted Protein Biomarker Quantitation Using LC-MS

Once biological samples are processed by any or combination of cleanup procedures, they can be subjected to “bottom-up” analysis, usually involving a series of steps leading to proteolytic digestion and LC-MS/MS analysis of selected peptides generated from the targeted analytes, or “top-down” analysis, direct measurement of the intact proteins. The former is the more commonly used approach for protein biomarker quantitation due to its better sensitivity.

#### 15.3.1 Surrogate Peptide Selection

The crucial steps in a “bottom-up” workflow are selection and quantitation of the surrogate peptides, as a representative of the targeted analyte protein. Trypsin is the most widely used protease in quantitation assays, and it can selectively cleave proteins at the carboxyl side of Lys and Arg unless Lys/Arg is followed by proline (Olsen et al., 2004). The surrogate tryptic peptides are much smaller than the intact protein. LC-MS analysis of peptides is more straightforward compared with that of intact protein, since LC separation and MS fragmentation can be challenging for large molecules. CID fragmentation of tryptic peptides generates predominately C-terminal y-ion and N-terminal b-ion series, which can be used in MRM scan for quantitative analysis (Coon, 2009).

Selection of a surrogate peptide is usually assisted by *in silico* digestion of a target protein biomarker with software such as Skyline, PeptideAtlas, and MRMAid, which produces a list of proteolytic peptides based on the protease of choice. A suitable enzyme can then be selected if it can generate peptides that cover specific regions of interest without homology with other proteins. Multiple proteases may also be used to improve digestion efficiency. Trypsin/Lys-C mixture, for instance, has been frequently used as an alternative of trypsin. Glatter et al. reported that the abundance of selected peptides generated by trypsin alone was 1.3–1.7 fold lower compared with trypsin/Lys-C mixture (Glatter et al., 2012). Combination of multiple proteases

(trypsin, LysC, ArgC, Asp-N, and Glu-C) was also described in a quantitative study of bacterial ribosomal proteins (Dator et al., 2014). Peptides with missed cleavage are usually avoided for protein quantitation. However, Chiva et al. evaluated the effect of including peptide with missed cleavages in protein quantitation and demonstrated that precision, accuracy, specificity, and sensitivity did not have significant difference compared with fully tryptic peptides (Chiva et al., 2014). Other recommendations for the selection of surrogate peptides include: (i) Select peptides that are large enough to achieve sufficient chromatographic retention and avoid homology with other proteins. Peptides with size between 500 and 2000 Da are recommended; (ii) Avoid peptides with reactive residues that are susceptible to chemical modification (Met oxidation, Trp oxidation, pyroglutamic acid formation, Asn and Gln deamidation, etc.); (iii) Avoid continuous sequence of Arg-Arg, Lys-Lys, Arg-Lys, or Lys-Arg, which may result in inconsistency in the formation of tryptic peptide (Li et al., 2011); (iv) Avoid peptides with Arg-Pro, Lys-Pro, and glycosylation site (Asn-X-Ser/Thr); (v) Select peptides that produce strong MS/MS signals for monitoring the transition. Rules for setting transitions are (i) precursor ions with +2 or +3 charge states; (ii) y series of fragment ions greater than y3 with +1 charge state; (iii) the five most intense fragment ions in the MS/MS spectra from untargeted analysis; (iv) *m/z* values of precursor and product ions between 300 and 1500 (Tsai et al., 2015). Multiple peptides derived from target protein or peptides can be simultaneously monitored in case of potential protein modification in a certain area (Cao et al., 2010). The number of transitions can be decided by dividing the duty cycle (normally 2–3 seconds) of the instrument by the dwell time (2–50 milliseconds) (Manes et al., 2015).

### 15.3.2 Sample Pretreatment Prior to Proteolytic Digestion

Sample pretreatment prior to protein digestion involves denaturation, reduction, and alkylation. Denaturation of the protein results in unfolding of the molecule, and makes the cleavage sites more accessible for proteases. Therefore, the digestion efficiency can be improved. One of the most common protein denaturation methods is the addition of strong chaotropic agents such as urea and guanidinium chloride at high concentration. However, these chaotropic reagents can also inhibit trypsin activity, and need to be diluted or removed before digestion (Chen et al., 2007). Surfactants such as RapidGest and ProteaseMax can also be used as alternative reagents for protein denaturation. These acid-degradable surfactants

are MS compatible, and prevent the additional steps for postdigestion extraction (Saveliev et al., 2013). Another convenient denaturation approach is the addition of organic solvent (usually acetonitrile or methanol) during trypsin digestion. Li et al. reported that the MS responses of tryptic peptides from myoglobin were enhanced by nearly fivefold using 50% methanol (Li et al., 2009). Reduction of proteins is commonly conducted by dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP). These reducing agents can break disulfide linkages, and subsequently make the cleavage site more accessible to proteases. Alkylation of the resulting cysteine-free thiol groups by iodoacetamide can prevent reformation of disulfide bonds. Dubois et al. argued that alkylation does not always make a significant difference in MS signal, and can be omitted to simplify the workflow (Dubois et al., 2008).

### 15.3.3 Proteolytic Digestion

The pretreated protein sample can then be digested by proteases in solution or immobilized on column. An enzyme to protein ratio of 1:20–1:100 (w/w) is commonly used which is determined by the amount of endogenous proteins after sample cleanup or enrichment processes. Generally speaking, the amount of protease can be significantly reduced when selective sample purification steps such as immunocapture are conducted (Li et al., 2009).

Although good sensitivity can be reached without purification after protein digestion, it is beneficial to perform a post-cleanup or enrichment to remove proteolytic peptides from interference proteins, detergents, and salts. These interferences may reduce the robustness of LC-MS. Generic methods such as PPT and SPE can be used for a rapid peptide cleanup (Lesur et al., 2010), while other selective approach such as immunocapture is proved to provide further improvement in sample cleanliness and sensitivity. Stable isotope standards and capture by anti-peptide antibodies (SISCAPA), first introduced by Anderson et al., is a powerful peptide enrichment strategy based on immunocapture of selected surrogate peptides (Anderson et al., 2004). Several highly sensitive LC-MS assays have been developed based on this method with sensitivity at low ng/mL (Whiteaker et al., 2007; Hoofnagle et al., 2008; Kuhn et al., 2009). SISCAPA also has unique advantages for tissue sample analysis due to its compatibility with harsh tissue extraction condition that may destroy binding epitope in the analyte protein. In addition, the potential interference for antiprotein capture such as membrane lipids, lipids from adipose tissues, or interference detergents used for tissue extraction can be bypassed by SISCAPA approach (Neubert et al., 2016).

Despite the advantages of SISCAPA, the application of this approach is limited due to the requirement of specific antibodies, which may take months to produce and develop.

#### 15.3.4 LC-MS Analysis

Separation of peptides generated by enzymatic digestion is often achieved by reversed-phase LC (RPLC), while in some cases, hydrophilic interaction chromatography (HILIC) is very useful to improve retention and ionization efficiency of highly polar peptides. In RPLC, the “rule of thumb” for column selection is that for small and hydrophilic peptides/proteins, longer bonded phase such as C18 or C12 should be used, while for large and hydrophobic proteins, shorter chains such as C4 is more optimal for achieving a better peak shape. Large pore size (e.g., 300 Å) should be chosen for large molecules, in order for the analyte to gain access to the surface chemistry of the stationary phase as well as to prevent clogging of the pore.

MRM on a triple quadrupole MS is the most common method for detection of the signature peptide. In an MRM experiment, the first quadrupole (Q1) is used to isolate the precursor ions of interest, and these ions are fragmented in the collision cell (Q2) to generate product ions by colliding with a collision gas. Then, product ions with predefined *m/z* values are detected by the second quadrupole analyzer (Q3). This two-stage mass filtering results in high specificity and sensitivity for MRM experiment. With current triple quadrupole instrument, more than a hundred transitions can be “monitored” in one single run, which gives LC-MS/MS based quantitation method multiplexing capabilities. In recent years, HRMS has also been increasingly used for targeted quantitation. HRMS in full scan mode can acquire accurate mass spectra of the analyte ions, which can be processed to generate extracted ion chromatogram (EIC) for quantitation. Alternatively, MRM<sup>HR</sup> mode, also named as parallel reaction monitoring (PRM), can be used for to acquire HRMS spectra of product ions from certain precursor ions that were selected in Q1 and specific product ions can be processed to generate EIC for quantitation with improved selectivity and sensitivity.

### 15.4 “Top Down” Approach for Targeted Protein Biomarker Quantitation Using LC-MS

While “bottom-up” approach remains the most common LC-MS-based method for macromolecule quantitation, there are several “top-down” approaches for protein

quantitation reported recently for solving the shortcomings of “bottom-up” methods. One limitation for “bottom-up” approach is that only parts of the target protein are quantified, and the concentration of the surrogate peptides may not represent the real concentration for the intact protein. Protein with similar sequences may produce same surrogate peptides, which limits specificity of the assays. “Bottom-up” method is also challenging when the biomarker is a protein with multiple PTMs, since the small tryptic peptides usually carry only one PTM. In addition, “bottom-up” method is not applicable for quantitation of protein isoforms or truncated forms. Different from “bottom-up” method, “top-down” approach directly detects the intact protein. Not only does it measure the real concentration of the biomarker in the biological matrices, but it also avoids the complicated sample preparation procedures in “bottom-up” workflow, such as proteolytic digestion, which may introduce artifacts and variations to the sample. It further decreases the method development time, because researchers do not have to consider issues related to the surrogate peptides.

Two main detection methods have been used on “top-down” approach for protein quantitation: MRM and HRMS. An example used MRM strategy for intact ubiquitin quantitation in cerebrospinal fluid. Ubiquitin has the ability to mark protein for degradation by the proteasome. The impairment of the ubiquitin-proteasome system is suggested to be an early event in the pathogenesis of neurodegenerative diseases such as Parkinson’s disease. Ubiquitin was purified by PPT using acetonitrile. The authors used the +12 charge state of ubiquitin and y58 fragment to set up the transition for quantitation. The method was validated in the range of 2–200 ng/mL with good intra- and interassay accuracy and precision (Oeckl et al., 2014).

With the advancement of MS technology, protein quantitation using ultra high resolution mass spectrometers gained much attention recently. HRMS is shown to provide better specificity, significant wide mass range, has the capability of quantifying proteins and their isomers in one experiment, and the potential for post-acquisition data mining. Compared with MRM-based approach, HRMS avoids fragmentation of the targeted molecule, which is quite challenging for proteins because of the poor fragmentation efficiency in CID (van den Broek and van Dongen, 2015). In one recent example, relative quantitation of different glycoisoforms of intact ApoC3 in human plasma was conducted by LC-HRMS. Apo C3 is one of the glycoproteins associated with disease states. Plasma samples were prepared by SPE, followed by LC-HRMS full scan analysis. Isotope peaks for each targeted glycoisoform at two charge states were

extracted using a window of 50 mDa and integrated into a chromatographic peak. The peak ratio of ApoC3-1/ApoC3-0 and ApoC3-2/ApoC3-0 was determined in a highly reproducible manner and has been found to be correlated to the diabetic status (Jian et al., 2013). Another research shows the usage of LC-MS for relative quantitation of histone H1 phosphorylation, which is suggested to be a clinical biomarker of breast cancer. In this case, “bottom-up” approach is not applicable, since the tryptic peptides are not useful for measuring the combinatorial PTM patterns on histone. The absolute abundance of each proteoform was determined by the highest intensity isotopic peak in the mass spectrum. The authors were able to quantify different proteoforms from cell lines MDA-MB-231 and MCF-10A using Fourier transform ion cyclotron resonance (FT-ICR) MS (Chen et al., 2016). When quantifying using isotopic peaks, a rule of thumb for HRMS analysis was given recently by Qian that the resolving power needs to be at least four times of the molecular weight of the protein for getting baseline separation of the isotopic peaks (Ruan et al., 2011). This limits the size of the proteins that can be analyzed by this approach using current HRMS technologies to 30 kDa. Alternatively, instead of using isotopic peaks to reconstruct EIC, the multiple charged peaks can be deconvoluted to mass spectra of zero charged peaks, which can be used for quantitation. This approach has been recently explored and showed great promise for quantitation of large proteins for which there is no separation between isotopic peaks (Jian et al., 2016). “Top-down” intact analysis of proteins in biological samples is still in its infancy and faces many challenges, the major one being lack of sensitivity. Multiple approaches, including advanced sample preparation such as immunoaffinity capture, “middle-down” analysis by first breaking the molecule into relatively smaller size, and miniaturized LC (micro-LC and nano-LC) for improved ionization efficiency are being explored to overcome the sensitivity issue.

## 15.5 Key Considerations in Targeted Protein Biomarker Quantitation Using LC-MS

### 15.5.1 Preanalytical Considerations

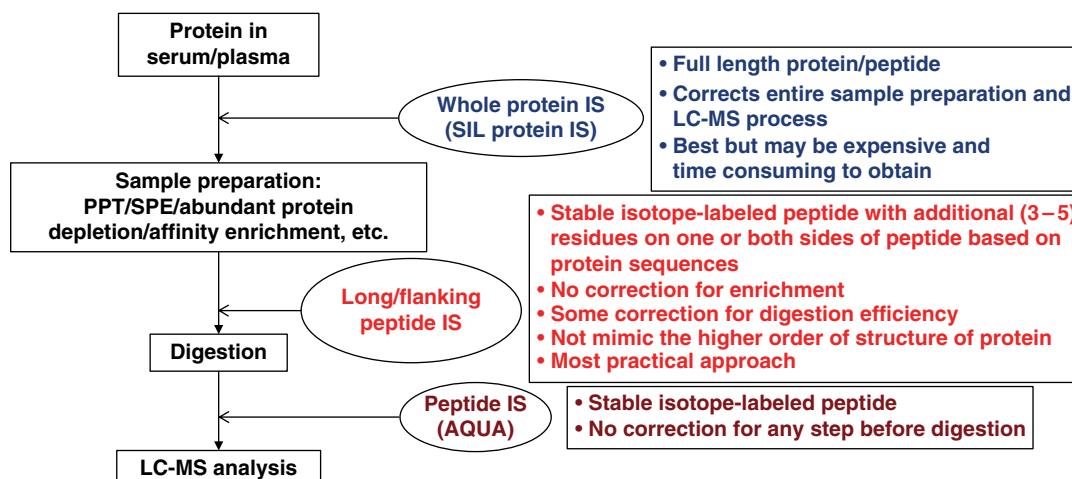
There are two main types of variability that can affect the truthfulness and reproducibility of measurement and therefore the validity of the biomarker data: preanalytical and analytical. Preanalytical variability refers to biological related factors such as intraindividual (e.g., diurnal, fasting state, day-to-day) and interindividual variability (e.g., age, gender, race, pregnancy, disease) and those occurring during sample collection and storage, while

analytical variability happens in the laboratory during sample analysis. Biological variability should be considered in the study design in order to produce statistically meaningful data. Sample collection and storage condition should be validated and standardized to maintain sample integrity and consistency in practice throughout the study (Lee and Hall, 2009). For protein biomarkers, maintaining stability is one of the key areas of preanalytical consideration. Details such as a type of venipuncture needle, duration of blood draw, and centrifugation condition could impact stability of targeted biomarkers. Forcing the blood through a narrow-bore needle or centrifuge at high speed could activate blood cells in the samples and cause artifact. Protease inhibitors should be added in samples promptly after blood draw to prevent ex vivo degradation of the targeted proteins. Samples should be processed and stored under controlled low temperature and avoid repeated freeze-thaw. For biological fluids of relatively low protein content (e.g., urine, cerebral spinal fluid), the sample collection and storage containers must be evaluated for nonspecific adsorption of the targeted protein to the surface. A common debate in the area of protein biomarkers is whether to use plasma or serum. Although serum is preferred for LBA because it is, in general, considered a “simpler” matrix, contains less proteins and no anticoagulant, Human Proteome Organization does not recommend serum for new proteomics studies due to the variable nature of the coagulation process and plasma has been found to be a more reproducible sample (Omenn et al., 2005; Omenn, 2007). For proteins that are involved in the coagulation pathway or platelet activation, they can only be quantified accurately in plasma.

### 15.5.2 Internal Standard

Due to the relatively complex workflow of LC-MS-based assay, variations may be introduced in any step during sample preparation, which can significantly diminish accuracy and precision of the assay. Fortunately, use of IS can be incorporated into LC-MS-based quantitation assay and a well-designed IS can track and compensate the variation of analytes to improve assay performance. Different types of IS that can be used in a typical “bottom-up” protein quantitation workflow is depicted in Figure 15.2.

The best IS for absolute quantitation of protein analytes is SIL protein IS (Bronsema et al., 2013). It is physically and chemically similar to the analyte, and therefore, sample enrichment and cleanup steps can be tracked. A review summarized different IS options for targeted quantification of protein biopharmaceuticals (Bronsema et al., 2012). Another paper summarized the advantages and pitfalls of using SIL protein IS (Pritchard et al., 2011).



**Figure 15.2** Internal standard strategies for protein biomarker quantitation using LC-MS.

Protein IS can be expressed in the presence of SIL amino acids in vitro using cell free systems (Brun et al., 2007), or in vivo using cell culture (Ong et al., 2002), a process known as stable isotope labeling by amino acid in cell culture (SILAC). SIL protein IS is suggested to be the most accurate and precise method, since it can minimize the differences in sample treatment and proteolytic digestion. Small protein IS can also be chemically synthesized (Jian et al., 2013).

SIL protein IS, however, may require substantial resources and a relatively long time to produce. Therefore, protein analog with similar structure as protein analyte can be used as an alternative IS. The selection of protein analog IS is usually based on the following consideration: (i) similar retention time of IS proteolytic peptides to that of surrogate peptides; (ii) similar recovery; (iii) absence of interference from analyte and other endogenous proteins (Olsen et al., 2004). Yang et al. reported using guanidinated protein as analog IS (GP-IS). It was prepared by converting arginine residues in protein analyte into homoarginine residues using guanidine. It is a rapid and cost-effective approach to produce a protein analog that has very similar functionality as the analyte (Yang et al., 2014).

A more straightforward IS strategy is SIL surrogate peptide IS, which usually can be synthesized at a lower cost compared with SIL protein IS. This so-called “AQUA” peptide (Gerber et al., 2003; Kirkpatrick et al., 2005) may be used for multiple protein drug candidates that share similar sequence, and therefore, can be beneficial for early drug development stage. Kuhn et al. used a <sup>13</sup>C-labeled surrogate peptide as IS to quantify CRP, a diagnostic marker of rheumatoid arthritis (Kuhn et al., 2004). In addition, a method for rapid production of SIL peptide IS using differential dimethyl labeling was also reported (Ji et al., 2009). SIL peptide IS is useful for tracking potential ion suppression. However, SIL peptide

IS does not track protein recovery and proteolytic digestion efficiency prior to LC-MS analysis. It is possible to result in high precision for protein quantitation. However, protein accuracy may not be determined correctly, because there could be selective protein loss during sample cleanup, and/or one or several labeled and nonlabeled peptides could undergo differential decomposition during digestion (Wang et al., 2015). Reproducibility of these steps must be demonstrated during method development.

To improve the performance of SIL peptide IS, scientists developed an alternative kind of IS called extended SIL peptide IS or flanking IS (Fernandez Ocana et al., 2012; Neubert et al., 2013; Faria et al., 2015). These peptides contain additional residues on one or both sides of the surrogate peptide. Extended SIL peptide IS can be digested along with the targeted protein. It generates the same surrogate peptide as the protein. Therefore, it can be used to monitor the digestion variability. If the workflow involves postdigestion peptide enrichment, extended SIL peptide IS can also be used to track variability and recovery of this step (Neubert et al., 2008).

### 15.5.3 Reference Standard

For LC-MS-based analysis of proteins, the criteria and consideration for choosing reference standards are very similar to those employed for IS. An intact protein should be chosen over a surrogate peptide because analyte loss during sample steps such as immunoaffinity capture, digestion, and ionization cannot be compensated by the peptide reference standard. There could be negative bias in the measured concentration when a surrogate peptide is used to prepare calibration samples, as shown in the example of quantifying myosin light chain 1, a biomarker of cardiac necrosis (Berna et al., 2007). Ideally, the

reference standard should be identical to the targeted analyte but it is rarely the case for protein biomarkers due to their heterogeneous nature, often existing in multiple forms. Biomarkers standard can be produced in pure form by synthesis or recombinant technology, which may not be the same as the endogenous forms. They can also be made by purification from biological samples but often lack defined quality. Because of above situation, majority of protein biomarker assays should be considered as relative quantitative assays where the reference standards are not well characterized, not available in a pure form, or not fully representative of the endogenous form.

#### 15.5.4 Improving Sensitivity of the Assay

Despite the rapid development of LC-MS instrumentation, sensitivity is still one of the potential drawbacks of LC-MS-based assay compared with LBA. Excellent sensitivity (pg/mL) can be easily achieved by LBA such as ELISA, while the sensitivity of LC-MS-based assay for proteins is usually in the high ng/mL to  $\mu\text{g}/\text{mL}$  range. Nevertheless, sample preparation techniques such as immunocapture have significantly improved detection limit of LC-MS assays. Combination of immunoaffinity capture at the protein level and the surrogate peptide level can significantly boost the sensitivity of the assay. For example, an impressive LLOQ of 0.78 pg/mL was obtained using sequential protein and peptide immunocapture as a double cleanup strategy for quantitation of interleukin 21 (IL21) in human and monkey serum (Palandra et al., 2013). A diagram of the work flow is illustrated in Figure 15.3.

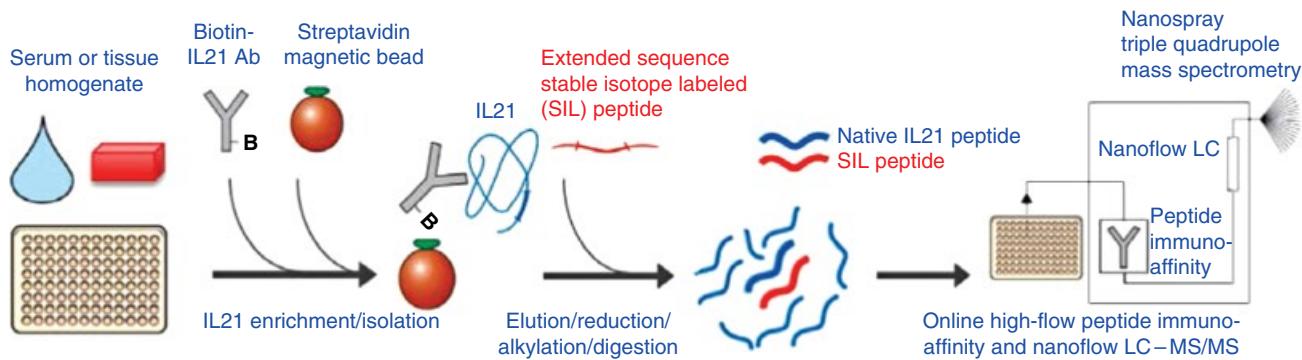
The need for gaining high ionization efficiency for improved sensitivity has pushed the application of capillary (column inner diameter of 0.1–0.5 mm, flow 1–20  $\mu\text{L}$ ) and nano-LC (column inner diameter < 0.1 mm, flow < 1  $\mu\text{L}/\text{min}$ ), though the sophisticated instrumentation and complicated operation have limited their application in routine sample analysis.

Adsorption loss of protein or peptide analytes resulted from nonspecific binding to surfaces may negatively affect sensitivity as well as linearity and repeatability of the assay (Maes et al., 2014). Therefore, strategies need to be developed to prevent sensitivity loss due to adsorption. Protein low-binding tubes or plates, which are commercially available, can also be used to minimize adsorption loss (Gaberc-Porekar et al., 2008).

#### 15.5.5 Improving Throughput of the Assay

The throughput of an LC-MS-based quantitative assay for large molecules can be limited due to the multiple steps in the workflow. In order to achieve high throughput, the length of each step needs to be reduced, and the number of samples that can be processed simultaneously needs to be maximized. In addition, automation can be employed for further improvement of the throughput and reproducibility. Sample cleanup and enrichment at both protein and peptide level can usually be performed in batch with the assistance of robotic liquid handlers. Common techniques such as PPT, SPE, nonspecific immunocapture, and depletion of abundant proteins can normally be done using 96-well plate format kits. For immunocapture that requires specific antibodies, sample and magnetic bead-processing can also be conducted by robotic liquid handlers (Neubert et al., 2013). Therefore, the enrichment or cleanup of 96 samples can be processed simultaneously within several hours and usually does not limit sample throughput.

Proteolytic digestion can also be performed in a plate format, but usually requires overnight incubation. Digestion can be performed more efficiently by protease at high concentration (8 mg/mL) and high temperature (60°C) in 1 hour (Ouyang et al., 2012). Other modern techniques such as microwave-assisted digestion (Berna and Ackermann, 2009; Lesur et al., 2010) and high pressure digestion (Lopez-Ferrer et al., 2008) can further reduce digestion time to 15 and 1 minute, respectively,



**Figure 15.3** Schematic of the IL-21 immunoaffinity LC-MS/MS assay workflow. Source: Palandra et al. (2013). Reproduced with permission of American Chemical Society.

but have not been developed into a 96-well plate format yet. Protease can also be immobilized on column for integrated online digestion and it facilitates digestion due to high enzyme:protein ratio in fixed space and reduced autolysis (Yuan et al., 2009). A pepsin-containing membrane was recently developed for monoclonal antibody digestion in 1 minute, which has potential for high throughput protein digestion (Pang et al., 2015). Immobilized enzymatic reactor (IMER) can be coupled with on-line immunocapture to provide a fully automated quantitative LC-MS assay of proteins in biological matrices (Hoos et al., 2006; Cingoz et al., 2010).

Multiplexed assay that can monitor multiple biomarkers in a single proteolytic sample is another effective approach to improve assay throughput. Whiteaker et al. demonstrated an automated and multiplexed method, where nine antipeptide antibodies were used for each sample to extract proteolytic peptides from nine different protein targets (Whiteaker et al., 2010). MRM methods were reported to quantify up to 27 cancer-biomarker proteins (Percy et al., 2013), and 142 noncommunicable diseases (NCDs) associated proteins (from 31 mg/mL to 44 ng/mL) in undepleted and nonenriched human plasma in a single run (Percy et al., 2014).

#### 15.5.6 Correlating MS Data with LBA Data

Because the final assay for large-scale clinical evaluation has been typically LBA, it is important to correlate between LC-MS assays and LBAs. For one study of correlation of concentration of carcinoembryonic antigen-related cell adhesion molecule 1 with patient survival, the LBA data indicated a significant correlation while MS data showed no significant difference. Because the protein has 11 isoforms, it is not known if both analytical methods targeted the same specific isoforms (Kalin et al., 2011). In another study of five proteins from plasma that had been previously shown to be overexpressed in pancreatic cancer tissue or pancreatic cell culture secretome, LC-MS assay for tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) was better than LBA for discriminating pancreatic cancer patients and the healthy controls. In this case, the actual concentrations measured by LC-MS were higher than those by LBA, indicating that LC-MS measured the total TIMP1 present in the plasma samples while LBA only detected the free form (Pan et al., 2012). There are also several other studies comparing LC-MS and LBA assays for protein biomarker quantitation in different disease areas such as lung cancer (Nicol et al., 2008), colon cancer (Lin et al., 2013), rheumatoid arthritis (Kuhn et al., 2004), and Down syndrome (Cho et al., 2011), in most of which LC-MS performed at least as well as LBA. Many factors such as specificity of the antibody used for LBA (and

LC-MS if immunoaffinity capture is employed), sample preparation procedure, and nature of the targeted proteins (isoforms, PTM, variants) could all impact correlation between the two platforms and should be taken into consideration when drawing conclusion.

## 15.6 Summary and Future Perspectives

LC-MS has been the workhorse of proteomics-based biomarker discovery and it is playing an increasingly important role in targeted protein quantitation for biomarker qualification, verification, and validation that leads to final clinical evaluation, where LBA has been the predominant platform. It is not expected that LC-MS will replace LBA but it has been proven to be a valuable complementary tool to LBA due to its unique advantages. For markers involving minor structure change in the protein, LC-MS can detect the changes more sensitively and specifically than LBA-based assays. For example, the formation of citrulline residues in proteins occurs by PTM of arginine and it has been identified as marker of inflammation. A robust MS assay has been developed to quantify citrullinated proteins in body fluid for detection of early-stage osteoarthritis (Ahmed et al., 2015). LC-MS can also readily detect and differentiate variants of protein biomarkers. There is a recent report on the detection of a previously undetected PSA variant coded by SNP-L132I. By using the specific MRM for the surrogate peptide from this variant, 9 samples out of 72 were found positive for this SNP and the results correlated well with a commercial immunoassay (Vegvari et al., 2013). For proteins existing in different isoforms that elicit different response in LBA, LC-MS is expected to provide a more straightforward calibration strategy. For example, the predominant form of PSA in serum is PSA-ACT (PSA bound to  $\alpha$ 1-antichymotrypsin) but other forms are also relevant including free PSA. LBA assays detect both free and PSA-ACT but the relative response to each form are different. Lack of standardization of the reference standards, particularly the ratio of the different forms, was recognized as contributing factors to disparities measured among different LBA assays (Chan and Sokoll, 2000). In contrast, LC-MS assay is usually a “total” assay where different forms can generate the same surrogate peptide, potentially providing a solution to the reference standard dilemma. Multiplexing is another area where LC-MS has advantage over LBA. It has been recognized that “one-protein one-disease” will not be the general rule due to the complex nature of diseases, and panels of biomarkers will be needed. MS is well suited to verification of large numbers of protein

biomarkers and for quantification of panels of biomarkers simultaneously. With advances in instrumentation, sample preparation technologies, and software, it is

expected that LC-MS will play a more and more important role in protein biomarker quantitation and become the gold standard platform for certain biomarkers.

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## 16

### Glycoprotein Biomarkers

*Shuwei Li<sup>1</sup>, Stefani N. Thomas<sup>2</sup>, and Shuang Yang<sup>2,\*</sup>*

<sup>1</sup> Institute for Bioscience and Biotechnology Research, University of Maryland College Park, Rockville, MD, USA

<sup>2</sup> Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, MD, USA

\* Current address: Center for Biologics Evaluation and Research, Office of Vaccine Research and Review, Laboratory of Bacterial Polysaccharides Vaccine Structure Group, FDA, Ellicott City, MD, USA

#### 16.1 Introduction

A biomarker, according to the National Institutes of Health (NIH) definition, is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic process, or pharmacologic responses to a therapeutic intervention” (Colburn et al., 2001). Alternatively, a broader definition of a biomarker is one or multiple biomolecules that are regulated or differentially expressed by diseased cells compared to normal cells. For example, gene expression patterns can be used as tumor biomarkers (Golub et al., 1999). Proteins, as well as their posttranslational modifications (PTMs), are also biomarkers that can indicate changes occurring beyond gene expression to provide the diagnosis of disease occurrence and progression (Hanash, 2003).

Biomarkers can be categorized into different types based on their applications, including diagnosis, prediction, prognosis, monitoring disease status, or monitoring disease response to therapy. Different types of biomarkers have been widely used in a variety of diseases. For example, protein biomarkers are produced either by the pathogen or by the host in response to pathological conditions. They could be used for screening and assessing a variety of diseases, such as cancers (Wulffkuhle et al., 2003; Visintin et al., 2008), cardiovascular diseases (Ridker, 2003; Danesh et al., 2004), diabetes (Pradhan et al., 2001), and infectious diseases (Allain et al., 1986; Simon et al., 2004). During diagnosis, protein biomarkers can be used for determining disease stages, selecting and evaluating therapy (Ludwig and Weinstein, 2005), and monitoring recurrent diseases during treatment (Peplow and Adams, 2015). Most proteins that have been used as biomarkers contain PTMs, such as

glycosylation (HER2 (Nahta et al., 2006), EGFR (Liu et al., 2011)), phosphorylation (MS4A1) (Almasri et al., 1992), and acetylation (KRAS) (Yang et al., 2012). Thus, it is of great significance to study posttranslationally modified proteins for biomarker discovery.

Protein glycosylation is one of the most common PTMs in addition to phosphorylation, acetylation, ubiquitination, nitrosylation, methylation, lipidation, and proteolysis. Glycosylation plays crucial biological and physiological roles in a living organism, from contributions to protein folding/quality control to involvement in a large number of biological recognition events (Moremen et al., 2012). Cell surface glycans affect their interactions with the extracellular environment by providing ligands for cell adhesion, macromolecule interactions, and pathogen invasion (Varki, 1993; Haltiwanger and Lowe, 2004). Glycans that are associated with protein receptors also modulate their functions and alter the dynamics of glycoprotein endocytosis through binding to multivalent lectins (Dennis et al., 2009). Changes in glycosylation are often a hallmark of disease states as aberrant glycosylation has been observed essentially in all types of experimental models of human diseases. For example, specific glycosylation may define cancer malignancy (Hakomori, 2002) as cancer cells frequently display glycans at different levels or with fundamentally different structures compared with those in normal cells (Dube and Bertozzi, 2005).

Protein glycosylation is a diverse and complex process arising from multiple factors (Stanley, 2011; Moremen et al., 2012). Glycan structures are highly heterogeneous due to their template-free biosynthesis, including variable compositions of different monosaccharides, different glycan branches, complicated linkages, and various isomeric forms (Daikoku et al., 2007). Glycosylation can

also occur on different sites, such as N-glycosylation on Asn, O-glycosylation on Ser/Thr, C-glycosylation on Trp (de Beer et al., 1995), and phosphoglycosylation on pSer/pThr (phosphoserine/phosphothreonine) (Haynes, 1998). N-glycosylation contains a core of five monosaccharide units that consist of two *N*-acetylglucosamines (GlcNAc) and three mannoses (Man). In comparison, O-glycans do not have a common core structure. Instead, they can be classified as mucin-type O-glycans that have eight different core structures containing an acetylgalactosamine (GalNAc) unit, or non-mucin-type O-glycans such as  $\alpha$ -linked O-fucose,  $\beta$ -linked O-xylose,  $\alpha$ -linked O-mannose,  $\beta$ -linked O-GlcNAc, or  $\alpha$ - or  $\beta$ -linked O-galactose (Kleene and Schachner, 2004; Tsuboi et al., 2012). In addition, N-linked glycans are conjugated to proteins through asparagine residues in the consensus tripeptide sequence Asn-X-Ser/Thr (or N-X-S/T, where X is any amino acid except proline) (Marshall, 1974), whereas O-linked glycans are conjugated to either serine or threonine residues that do not have an apparent consensus sequence domain (Wells et al., 2001; Whelan et al., 2008; Brockhausen et al., 2009). The analysis of glycosylation is thus inherently complicated by various glycosylation sites and their associated complex glycans. Numerous studies have been devoted to developing technologies to analyze glycosylation. In this chapter, we summarize the common technologies for glycoprotein analysis and discuss the roles of glycoproteins in diseases.

## 16.2 Technologies for Glycoprotein Analysis

Glycoprotein analysis involves a broad range of techniques for sample preparation, assignment of glycan structures, identification of glycosylation sites, determination of glycopeptide sequences, and quantification. Sample preparation (e.g., enrichment of glycopeptides and glycoproteins) can be performed using chemoenzymatic approaches or lectin affinity chromatography, whereas identification is usually achieved by mass spectrometry (MS), fluorescence spectrometry, or UV/VIS spectroscopy. Many of these techniques are compared here based on their advantages and limitations.

### 16.2.1 Glycoprotein Enrichment

#### 16.2.1.1 Techniques for the Enrichment of Glycoproteins

Glycoproteins can be enriched by chemical immobilization (Zhang et al., 2003) or lectin affinity column chromatography (Kaji et al., 2003). Chemical immobilization requires oxidation of glycans in order to conjugate

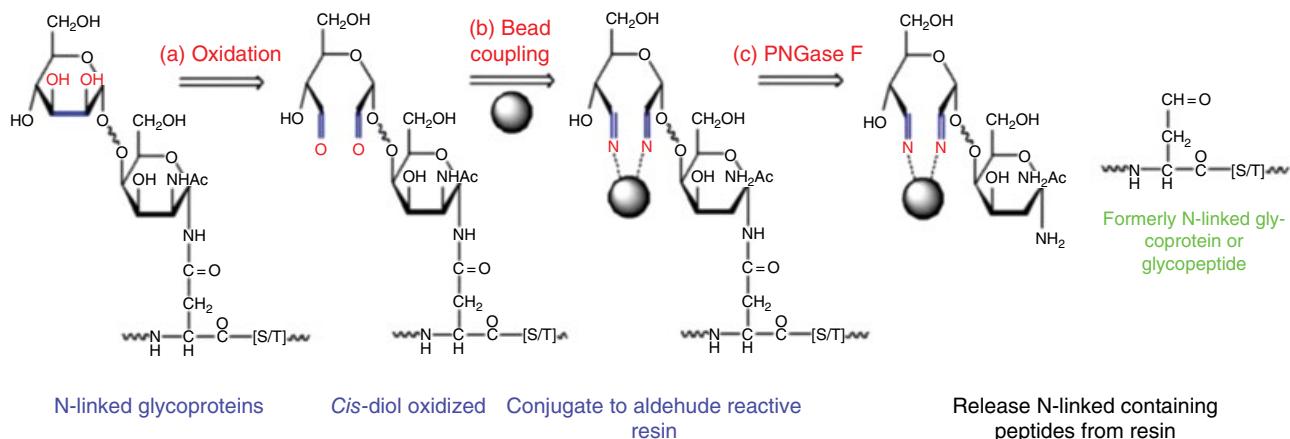
glycoproteins on solid support beads while non-glycosylated proteins are removed by a simple washing step. On the other hand, lectin affinity column chromatography takes advantage of the inherent binding between lectins and glycans. The former is highly specific for glycoprotein enrichment but destroys glycans during the enrichment process. The latter is less specific, yet it can keep glycoproteins intact.

**16.2.1.1.1 Chemoenzymatic Enrichment** Glycoproteins have glycans conjugated on their amino acids such as asparagine (N-linked) or serine/threonine (O-linked). One chemoenzymatic approach uses hydrazide chemistry to enrich glycoproteins or glycopeptides. As shown in Figure 16.1, glycoproteins are enriched by solid-phase extraction of glycopeptides (SPEG) (Zhang et al., 2003). The *cis*-diol of a glycan conjugated to asparagine (N) is first oxidized by an oxidizer such as sodium periodate ( $\text{NaIO}_4$ ) to form aldehydes, which subsequently react with hydrazide groups immobilized on a solid support to form covalent hydrazone bonds. The non-glycosylated proteins, or glycoproteins that do not have a *cis*-diol group, are removed by washing and collected for the optional analysis of non-glycoproteins. The conjugated glycoproteins or glycopeptides are then released by PNGase F for further analysis.

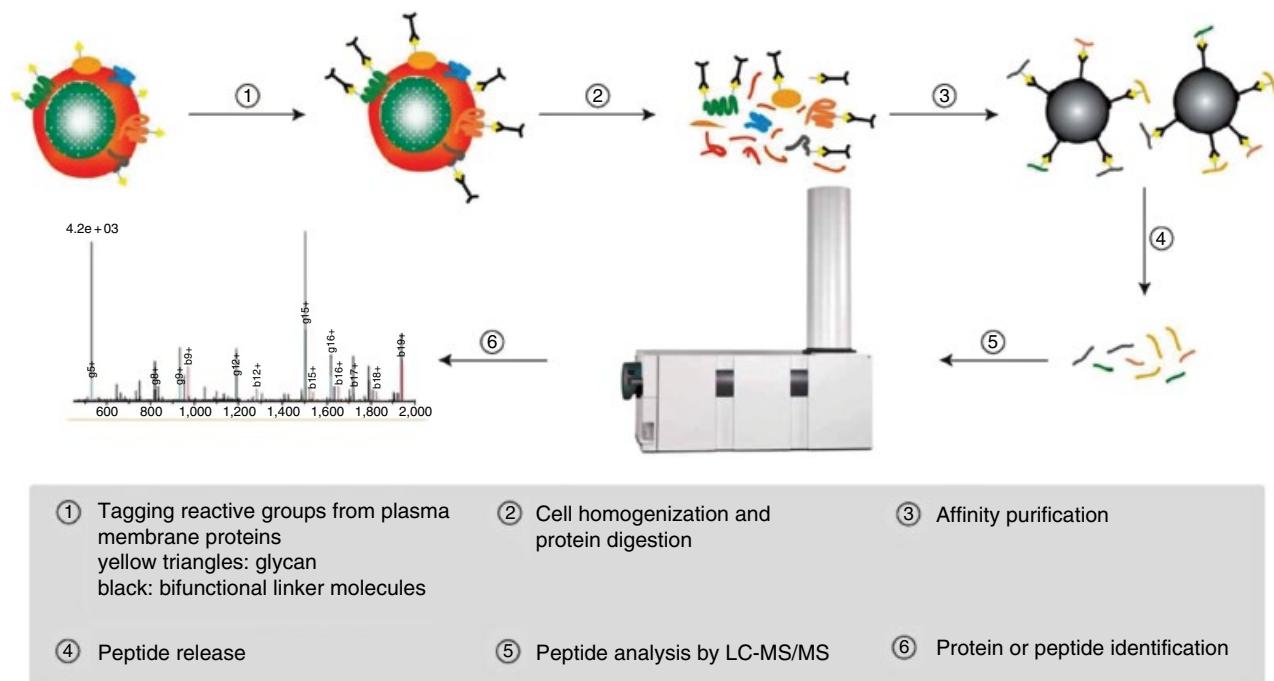
One may expect that glycopeptide enrichment using SPEG at peptide level would outperform glycoprotein enrichment at protein level. A glycoprotein immobilized on the solid support may consist of only one or several glycosylation sites. Upon trypsin digestion, most resulting peptides are non-glycosylated, which could interfere with the identification of less abundant glycopeptides. However, recent studies revealed that SPEG at protein level results in much higher identification and specificity (Berven et al., 2010; Wang et al., 2011b), even though SPEG at peptide level has better reproducibility. For example, a mouse plasma study demonstrated that the number of unique glycopeptides identified from the protein-level SPEG was threefold higher than the number obtained from the peptide-level SPEG (Wang et al., 2011b). This unexpected better performance of the protein-level SPEG could be attributed to two aspects: (i) the better capture efficiency of glycoproteins since they often carry multiple glycosylated sites on the same protein (Wang et al., 2011b) or (ii) the loss of highly hydrophilic glycopeptides during cleanup prior to hydrazide bead coupling when peptide-level SPEG is performed (Berven et al., 2010). Nevertheless, despite the low identification rate, the reproducibility of the peptide-level SPEG, when coupled with LC-MS/MS analysis, is high enough to be used for discovery-based clinical proteomics.

Another chemoenzymatic technique takes advantage of the biotin tag, whose binding with streptavidin is the

### SPEG: solid-phase extraction of glycopeptides



**Figure 16.1** Schematic diagram for the enrichment of glycosylated peptides/proteins. Solid-phase extraction of glycopeptides/glycoproteins (SPEG) utilizes the *cis*-diol of carbohydrates for the selective enrichment of glycopeptides/glycoproteins. The immobilized glycopeptides/glycoproteins are released by PNGase F digestion. Source: Zhang et al. (2003). Reproduced with permission of Nature Publishing Group.



**Figure 16.2** A strategy for capturing cell surface glycoproteins using a multistep tandem affinity labeling strategy (Wollscheid et al., 2009). The steps involve (1) tagging reactive groups from proteins, (2) cell homogenization and protein enzymatic digestion, (3) affinity purification, (4) peptide release, (5) LC-MS/MS, and (6) protein or peptide identification. Source: Wollscheid et al. (2009). Reproduced with permission of Nature Publishing Group.

tightest noncovalent interaction existing in nature, for the highly specific analysis of glycosylated membrane proteins (Wollscheid et al., 2009). As shown in Figure 16.2, carbohydrates on cell surface proteins are oxidized to carbonyl groups, which are conjugated with a

bifunctional linker containing a carbonyl-reactive hydrazide and a biotin moiety. The presence of the biotin tag allows glycopeptides to be immobilized on streptavidin beads with high efficiency and specificity. Enzymatic (PNGase F or A) treatment then releases these enriched

peptides from the streptavidin beads for their identification by LC-MS/MS. This technology, named cell surface capturing (CSC), enables the comprehensive and quantitative analysis of cell surface glycoprotein landscape (Wollscheid et al., 2009). It has recently been applied for the in-depth study of cell surface glycoproteins related to multidrug resistance in gastric cancer (Li et al., 2013), demonstrating its great value for clinical studies.

**16.2.1.1.2 Affinity Enrichment** Lectin affinity chromatography has been a popular tool for glycoprotein isolation since the early 1980s (Cummings and Kornfeld, 1982). Lectins are carbohydrate-binding proteins that play numerous roles in biological recognition events involving cells, carbohydrates, and proteins (Rutishauser and Sachs, 1975; Brudner et al., 2013). More importantly, interactions between lectins and their binding carbohydrates can be disrupted by specific mono- and/or oligosaccharides through a competitive binding mechanism, thereby allowing the enriched glycoproteins to be released under native conditions.

Table 16.1 summarizes lectins commonly used for glycoprotein enrichment (Zatta and Cummings, 1992). These carbohydrate-binding proteins are mostly extracted from plants and can be conjugated to the solid support through their amino acids, such as lysine/N-terminus, cysteine, aspartic acid/glutamic acid/C-terminus, and serine/threonine (Yang et al., 2013a, 2016). The solid support often uses a porous agarose matrix that provides a high surface-to-volume ratio for lectin conjugation (Turkova, 1999).

The general strategy for glycoprotein affinity enrichment is shown in Figure 16.3. It consists of lectin affinity capture, proteolysis, glycopeptide enzymatic release, and 2D-LC-MS/MS (Kaji et al., 2003). This method, termed isotope-coded glycosylation site-specific tagging (IGOT), is based on the lectin column-mediated affinity capture of glycoproteins from protein mixtures (Kaji et al., 2003). In the IGOT method (Figure 16.3a, b), glycoproteins are first captured by lectin affinity chromatography from all proteins extracted from a biological sample, which are then digested by trypsin to produce a mixture of peptides. PNGase F is added in the presence of isotope-coded H<sub>2</sub><sup>18</sup>O (heavy oxygen), which removes N-glycans from Asn and allows <sup>18</sup>O to be incorporated into deglycosylated peptides to generate <sup>18</sup>O-tagged Asp, as shown in Figure 16.3b. The resulting 3 Da (<sup>18</sup>OH vs. NH<sub>2</sub>) mass shift enables the unambiguous assignment of the glycosylation sites by MS/MS analysis. This approach has been widely used for studying protein glycosylation in many disease models (Hägglund et al., 2004; Wollscheid et al., 2009). Recent studies have also been devoted to the development of methods for glycopeptide enrichment and separation in order to improve glycosylation analysis in complex biological samples.

Serum is a valuable body fluid as serum proteins are routinely used for disease diagnosis and prognosis (Petricoin et al., 2002). However, serum contains over 20 high abundance proteins (e.g., the six most abundant proteins represent roughly 85% of the total human serum protein mass (Echan et al., 2005)) that greatly suppress the identification of low abundance glycoproteins (Anderson and Anderson, 2002). To analyze serum glycoproteins, the depletion of these high abundance proteins is usually required. Lectin affinity chromatography is therefore an ideal tool for the analysis of serum glycoproteins (Figure 16.3c) (Drake et al., 2006) because they can be directly captured by the lectins without the prerequisite to remove high abundance proteins. Alternatively, after the depletion of high abundance proteins, serum proteins can be digested by trypsin, and glycopeptides can be enriched by lectin columns for further analysis. These sample preparation protocols have been integrated well with an immunoaffinity-based targeted analytical approach (Drake et al., 2006), which enables us to carry early discovery studies through confirmation and rapid pre-validation, prior to the expensive and time-consuming process of raising glyco-specific antibodies.

Lectin affinity chromatography can be employed as an array platform to accommodate high-throughput glycoprotein detection (Wilson and Nock, 2003; Patwa et al., 2010). Instead of using a single lectin, tens of lectins are immobilized on a glass slide for the simultaneous monitoring of hundreds of reactions since different lectins can recognize specific glycan structures, including acetylgalactosamine, mannose, galactose, fucose, sialic acid (Neu5Ac or Neu5Gc), and acetylglucosamine. Specific glycoprotein antibodies can also be used in an array format, enabling global profiling of glycoprotein expression for biomarker discovery (Wilson and Nock, 2003).

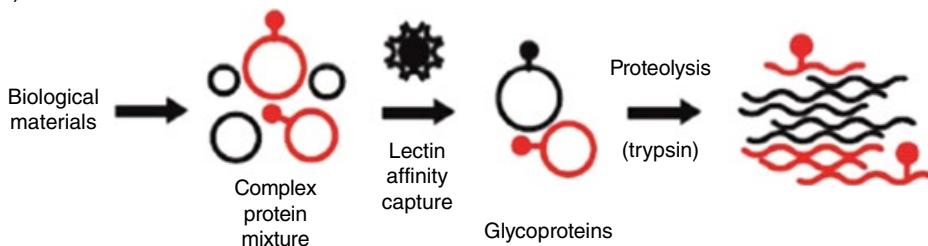
### 16.2.1.2 Hybrid Chemical Metabolic Labeling

Metabolic labeling is another popular method in which an unnatural tag is incorporated into various biomolecules during their biosynthesis. For example, mucin-type O-linked glycan biosynthesis is initiated by polypeptide N-acetylgalactosamine transferase (ppGalNAcT) in the presence of uridine 5'-diphospho-N-acetylgalactosamine (UDP-GalNAc), which is the high energy donor of GalNAc unit during polysaccharide biosynthesis (Hang et al., 2003). This enzyme can also take up an UDP-GalNAc analog carrying an azido group (GalNAz) as a substrate and incorporate this nonnative monosaccharide unit into O-linked glycans. The azido group can couple with a biotinylated phosphine probe through the specific bio-orthogonal Staudinger reaction (Figure 16.4) (Saxon and Bertozzi, 2000) for subsequent enrichment and detection. Recently, a similar approach called IsoTaG

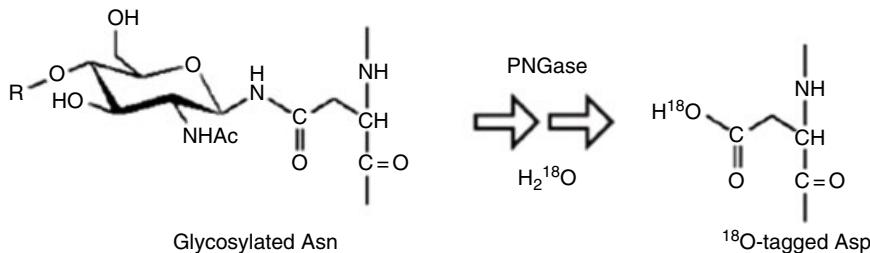
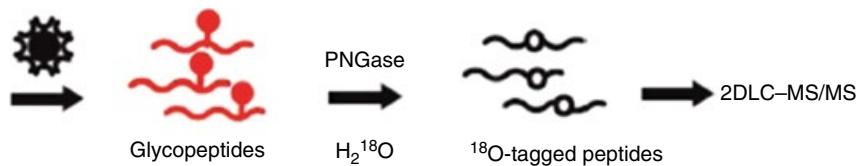
**Table 16.1** Common lectins used for glycoprotein enrichment.

Symbol	Description	Source	Ligand motif	Glycan
UEA	Ulex europaeus agglutinin	<i>Ulex europaeus</i>	Fuc $\alpha$ 1-2Gal-R	Fucose
AAL	Aleuria aurantia lectin	<i>Aleuria aurantia</i>	Fuc $\alpha$ 1-2Gal $\beta$ 1-4(Fuc $\alpha$ 1-3/4)Gal $\beta$ 1-4GlcNAc, R2-GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-6)GlcNAc-R1	Fucose
RCA	Ricin, ricinus communis agglutinin, RCA120	<i>Ricinus communis</i>	Gal $\beta$ 1-4GalNAc $\beta$ 1-R	Galactose/GalNAc
PNA	Peanut agglutinin	<i>Arachis hypogaea</i>	Gal $\beta$ 1-3GalNAc $\alpha$ 1-Ser/Thr (T-antigen)	Galactose/GalNAc
AIL	Jacalin	<i>Artocarpus integrifolia</i>	(Sia)Gal $\beta$ 1-3GalNAc $\alpha$ 1-Ser/Thr (T-antigen)	Galactose/GalNAc
VVL	Hairy vetch lectin	<i>Vicia villosa</i>	GalNAc $\alpha$ -Ser/Thr (Tn antigen)	Galactose/GalNAc
ABL	Agaricus bisporus lectin	Basidiomycete mushroom	Gal $\beta$ 1-4GalNAc $\beta$ (T antigen)	Galactose/GalNAc
WGA	Wheat germ agglutinin, WGA	<i>Triticum vulgaris</i>	GlcNAc $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc, Neu5Ac (sialic acid)	GlcNAc
ConA	Concanavalin A	<i>Canavalia ensiformis</i>	$\alpha$ -D-Mannosyl and $\alpha$ -D-glucosyl residues branched $\alpha$ -mannosidic structures (high $\alpha$ -mannose-type or hybrid-type and biantennary complex-type N-glycans)	Mannose
LCH	Lentil lectin	<i>Lens culinaris</i>	Fucosylated core region of bi- and triantennary complex-type N-glycans	Mannose
GNA	Snowdrop lectin	<i>Galanthus nivalis</i>	$\alpha$ 1-3- and $\alpha$ 1-6-linked high mannose structures	Mannose
SNA	Elderberry lectin	<i>Sambucus nigra</i>	Neu5Ac $\alpha$ 2-6Gal(NAc)-R	Neu5Ac
MAH	Maackia amurensis hemagglutinin	<i>Maackia amurensis</i>	Neu5Ac/Gca2,3Gal $\beta$ 1,3(Neu5Ac $\alpha$ 2,6)GalNac	Neu5Ac
MAL	Maackia amurensis leukoagglutinin	<i>M. amurensis</i>	Neu5Ac/Gca2,3Gal $\beta$ 1,4Glc(NAc)	Neu5Ac/Neu5Gc
PAL	Pterocarpus angolensis lectin	<i>Pila globosa</i> snail	Neu5Gc	Neu5Gc

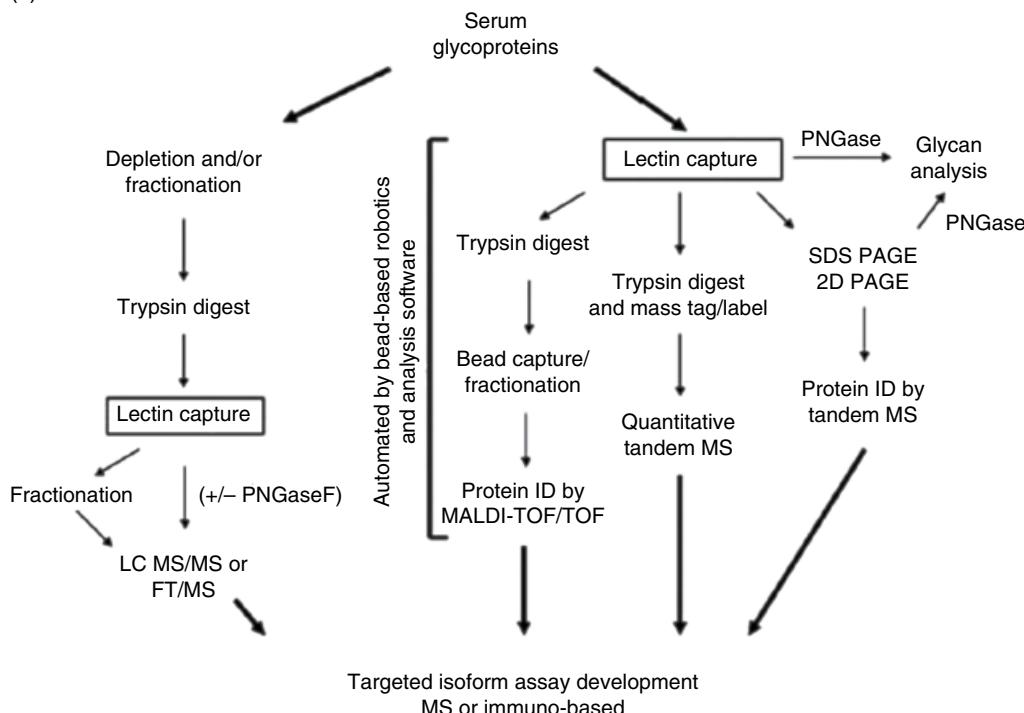
(a)



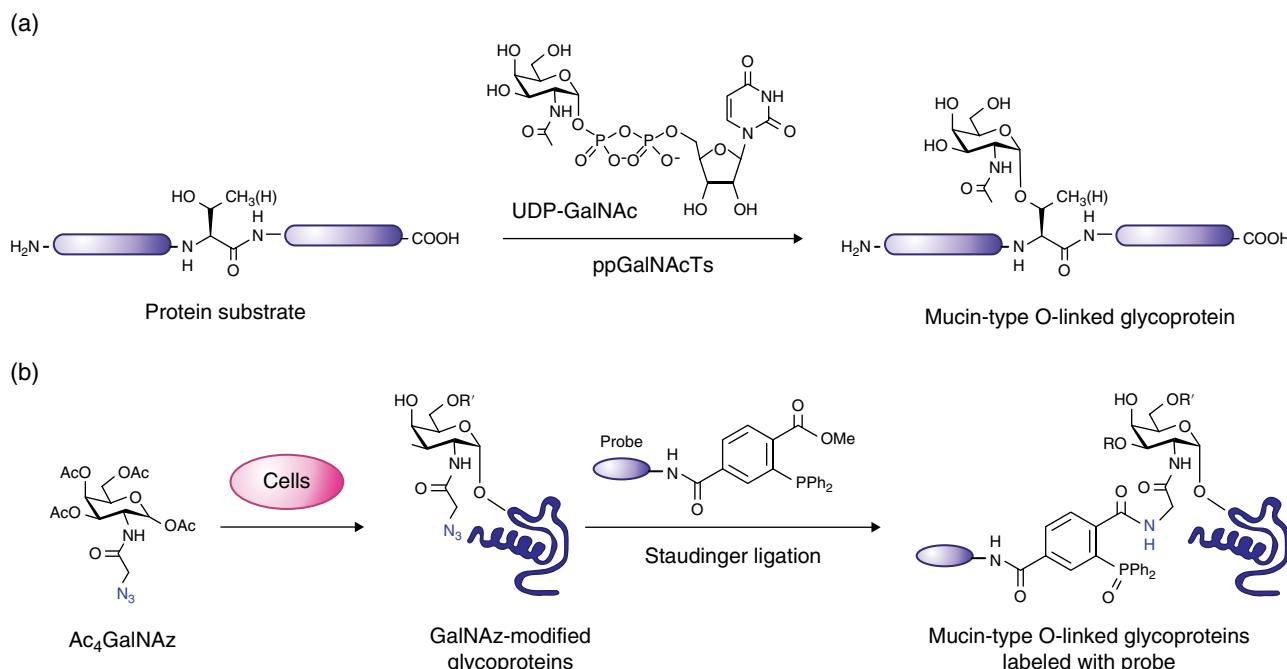
(b)



(c)



**Figure 16.3** Schematic representation of the lectin affinity enrichment of glycoproteins for mass spectrometry. (a) Enrichment of glycoproteins by lectin affinity chromatography. (b) Release of glycoproteins by PNGase F digestion. Source: Kaji et al. (2003). Reproduced with permission of Nature Publishing Group. (c) Targeted isoform assay by the combination of lectin affinity chromatography and traditional gel electrophoresis. Source: Drake et al. (2006) <http://www.mcponline.org/content/5/10/1957.short>. Used under CC BY 4.0 <https://creativecommons.org/licenses/by/4.0/>.



**Figure 16.4** Metabolic labeling approach for the proteomic analysis of mucin-type O-linked glycosylation using bio-orthogonal Staudinger reaction between azides and phosphines. (a) Initiation of mucin-type O-linked glycan biosynthesis by the ppGalNAcTs. UDP-GalNAc is the nucleotide sugar donor substrate for the ppGalNAcT family. (b) Strategy for metabolic labeling of mucin-type O-linked glycoproteins with an azido GalNAc analog (GalNAz) for proteomic analysis with phosphine probes. Source: Hang et al. (2003). Reused with permission of National Academy of Sciences.

(isotope-targeted glycoproteomics) was developed for the study of intact, metabolically labeled N-linked and O-linked glycopeptides at the whole proteome scale (Woo et al., 2015). This method is based on click chemistry, another bio-orthogonal reaction between azide and alkyne groups, to attach a biotin tag onto glycoproteins. A unique feature of IsoTaG is its inclusion of two bromine atoms, whose characteristic isotope pattern allows tag-containing glycopeptides to be searched and identified from full MS spectra with high confidence (Woo et al., 2015).

### 16.2.2 Glycan Analysis

Glycans are defined by the International Union of Pure and Applied Chemistry (IUPAC) as “compounds consisting of a large number of monosaccharides linked glycosidically,” which includes N-linked or O-linked types, as well as glycosaminoglycans such as heparin, heparan sulfate, and so on. This section focuses on methods for the analysis of N-linked and O-linked glycans.

#### 16.2.2.1 In-Solution Glycan Analysis

To analyze glycans, their release from glycoproteins by enzymatic digestion or chemical reactions is often needed. Several enzymes are available for the release of N-glycans (Table 16.2), including PNGase F, PNGase A,

Endo D, Endo H, and Endo F, while only one O-glycosidase can be used to release Core-1 and Core-3 disaccharides O-glycans. These enzymes cleave glycans at specific oligosaccharide sites. For example, PNGase F digests most N-glycans from either short glycopeptides or intact glycoproteins except those containing an α-1,3 core fucosylated GlcNAc (Tretter et al., 1991); PNGase A cleaves all N-glycans, but only from short glycopeptides (Plummer and Tarentino, 1981); and Endo D, Endo H, and Endo F cleave the linkage between the first and second GlcNAc units of N-glycans. The specificities of these enzymes are summarized in Table 16.2.

A chemical method, oxidative release of natural glycans (ORNG), has been reported for the release of different types of glycans (Song et al., 2016). An oxidant, sodium hypochlorite (NaClO), has been used to effectively and selectively degrade the aglyco portion of native glycoconjugates to release intact glycans (Hawkins and Davies, 1998), which can be further derivatized through their reducing end. The release of O-linked glycans requires prolonged treatment by NaClO, given the fact that O-glycosidic linkages are more stable than N-glycosides.

The structural analysis of glycans is often achieved by permethylation, in which all hydroxyl groups (–OH) are derivatized to methoxy groups (–OCH<sub>3</sub>) (Ciucanu and Kerek, 1984). Permethylated glycans are more

**Table 16.2** Common glycosidases for *N*-glycan release and chemicals for *O*-glycans.

Enzymes or chemicals	Cleavage site	Details	Type
PNGase F	Asn- $\beta$ -GlcNAc	$\alpha$ 1,3 core fucosylated cannot be cleaved	N-linked
PNGase A	Asn- $\beta$ -GlcNAc	All	N-linked
Endo D	Asn-GlcNAc- $\beta$ -GlcNAc	C2 of $\alpha$ 1,3-linked core mannose, hybrid, or complex cannot be cleaved	N-linked
Endo H	Asn-GlcNAc- $\beta$ -GlcNAc	$\alpha$ 1,6-linked mannose must be substituted with one or more mannose residues	N-linked mannose and hybrid
Endo F (1,2,3)	Asn-GlcNAc- $\beta$ -GlcNAc	Cleave most high mannose and biantennary hybrid oligosaccharides. Tri- and tetraantennary chains cannot be cleaved, not a bisected biantennary chains	N-linked mannose and biantennary
O-glycosidase	Ser/Thr- $\beta$ -GalNAc	Only cleave core-1 and core-3	O-linked
$\beta$ -elimination	Ser/Thr- $\beta$ -(GalNAc, GlcNAc, Glucose), Asn- $\beta$ -GlcNAc	Cleave most O-linked glycans and partially release N-linked glycans, but peeling occurs without chemical protection	O-linked, N-linked
Bleach (Song et al., 2016)	Asn- $\beta$ -GlcNAc; Ser/Thr- $\beta$ -(GalNAc, GlcNAc, mannose, glucose); carbohydrate- $\beta$ -ceramide	Cleave most types of glycans	All glycans

stable and hydrophobic, improves their MS ionization responses, and generates fragment ions with greatly improved MS/MS signals. Due to these important aspects, permethylation has been employed extensively for the determination of glycan structures and linkages (Sheeley and Reinholt, 1998).

#### 16.2.2.2 Solid-Phase Glycan Analysis

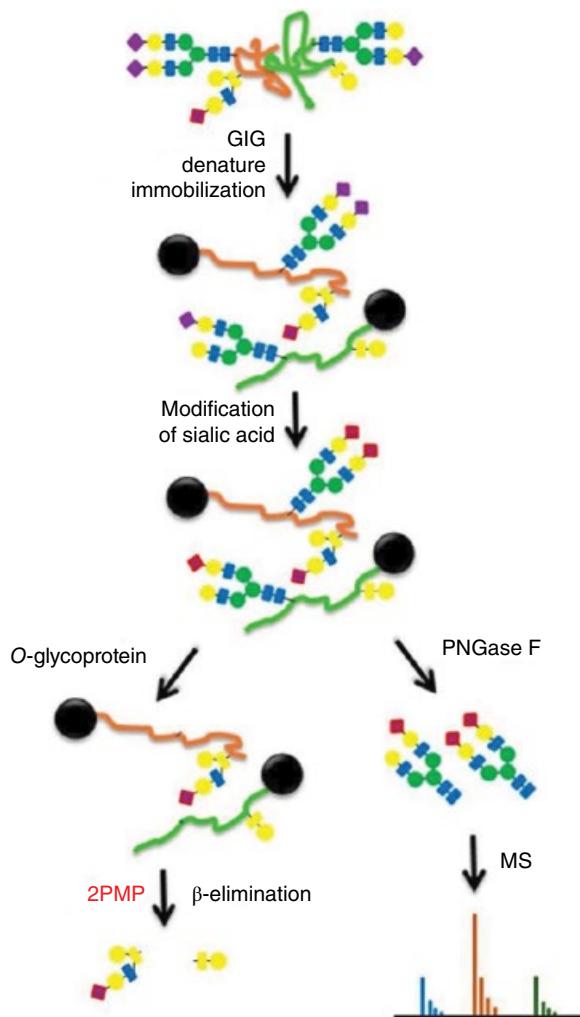
Glycan analysis on solid support is an emerging technology that presents unique advantages for sample preparation (Yang and Zhang, 2012; Yang et al., 2013a, 2013b). For example, the analysis of sialic acids by MS is challenging, mainly owing to the fragile nature of these species (Zaia, 2010). Glycoproteins from serum often contain highly sialylated N-linked and O-linked glycans (Morelle and Michalski, 2007; Royle et al., 2008; Aldredge et al., 2012). Without stabilization, sialic acids are partially or completely lost during matrix-assisted desorption/ionization (MALDI)-MS (Sekiya et al., 2005). Permetylation is commonly used to stabilize sialic acids by converting their hydroxyl to methoxy groups. However, it is not an ideal method for the analysis of sialoglycans containing acetylated or methylated sialic acids (Varki, 1992; Kohla et al., 2002) because *O*-acetyl groups are decomposed under harsh permethylation conditions, whereas partially methylated sialic acids are masked by derivatization. Methyl esterification or amidation can selectively modify sialic acids under much milder conditions, thus keeping glycans or proteins intact (Toyoda et al., 2008). However, it is difficult to remove chemical compounds after these

modifications are introduced when the reaction is performed in solution (Yang et al., 2013a; Yang and Zhang, 2014). Solid-phase-based approaches can solve such dilemma and greatly simplify sample preparation.

Glycoprotein immobilization for glycan extraction (GIG) (Yang et al., 2013a) is a technique to mitigate these difficulties. Figure 16.5 shows the schematic workflow of GIG for the analysis of *N*-glycans and *O*-glycans. Proteins are denatured completely and immobilized covalently onto aldehyde-functionalized resin through N-termini or lysines by reductive amination. Sialic acids on glycoproteins are then modified with *p*-toluidine on solid support beads, which makes it convenient to use excess reagent for the complete amidation of sialic acids. *N*-glycans are then released by PNGase F treatment, while *O*-glycans are cleaved by base-induced  $\beta$ -elimination (Wang et al., 2011a) for their structural determination. Therefore, this approach allows both N-linked and O-linked glycans from the same specimen to be analyzed.

#### 16.2.3 Automated Platform for Processing Clinical Specimens

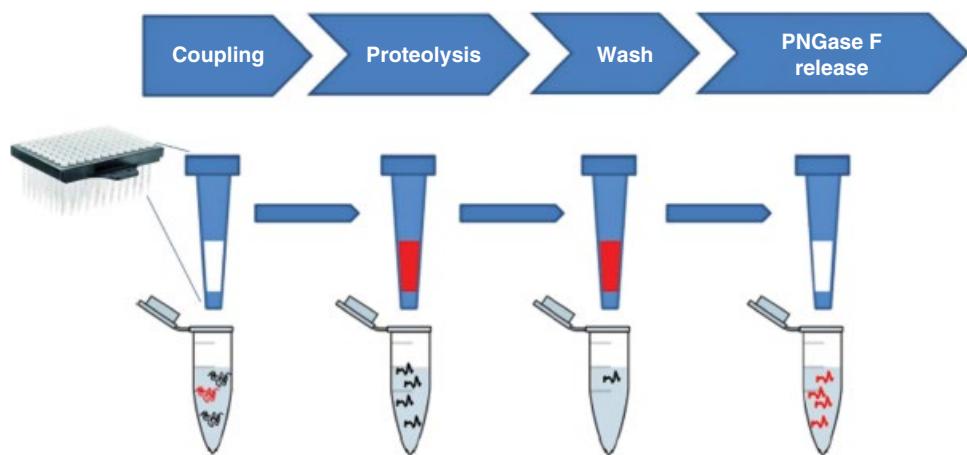
Sample preparation is usually the first step for glycoprotein analysis. It is essential to have a robust, high-throughput, and reproducible platform to generate high quality samples. As discussed previously, sample preparation can be conducted using an in-solution approach or using a solid support. The compatibility of solid-phase-based approaches with high-throughput



**Figure 16.5** Chemoenzymatic solid-phase method for the analysis of N-linked and O-linked glycans using GIG. Source: Yang et al. (2013a) and Yang and Zhang (2014). Reprinted with permission of American Chemical Society.

platforms has made this approach incredibly attractive for glycoprotein and glycan sample preparation. For example, glycopeptides can be purified by cotton hydrophilic interaction liquid chromatography (HILIC) micro-tips (Selman et al., 2011) or Sepharose HILIC SPE in a 96-well plate format (Selman et al., 2010). Even though these techniques have already improved sample preparation with better reproducibility and yield, considerable manual operations are still required.

A novel platform has been reported recently for the analysis of glycopeptides using SPEG (Chen et al., 2013), which is a solid-phase-based approach for glycopeptide enrichment. The procedure includes several steps: (i) conjugate oxidized glycoproteins onto hydrazide-coated beads; (ii) perform trypsin digestion of immobilized proteins; (iii) wash away non-glycosylated tryptic peptides; and (iv) release glycopeptides with PNGase F treatment (Figure 16.6). Instead of performing these steps manually, hydrazide beads are packed in microscale pipette tips, which allows all steps to be carried out by an automated liquid handler (such as Thermo Versette or Agilent Bravo). Briefly, proteins from biological samples are oxidized by sodium periodate, followed by buffer exchange into the coupling buffer. Proteins are then dispensed into a 96-well plate and aspirated into hydrazide tips continuously for multiple rounds. This method has unique advantages since samples are forced to pass through the bead surface and their inner pores, significantly improving the coupling efficiency and reducing sample preparation time. Complete coupling can be achieved within 20–30 min, and the overall processing time has been reduced from 3 to 4 days using the manual procedure to 8 h using an automated process (Chen et al., 2013). Therefore, such automated platform has great potential for processing a large number of samples, especially for clinical diagnosis.



**Figure 16.6** Schematic workflow of N-linked glycopeptide isolation using hydrazide tip by an automated liquid handler. Source: Chen et al. (2013). Reproduced with permission of American Chemical Society.

### 16.2.4 MS Analysis of Glycoproteins

Glycoproteins are often analyzed by bottom-up or top-down MS-based approaches. In bottom-up approaches, glycoproteins are enzymatically digested prior to MS analysis. With previously known glycans and glycosites, intact glycopeptides are searched by spectral matching or spectral library matching. In top-down methods, glycoproteins are directly analyzed by MS and may be compared with PNGase F-treated deglycosylated proteins.

#### 16.2.4.1 Bottom-Up Approaches

Bottom-up proteomics is widely used to identify protein sequences and PTMs. In this approach, glycoproteins are digested by proteases prior to MS analysis. Its advantages include the ability to achieve high-resolution separations and comprehensive coverage of proteins from a limited amount of samples. In conjunction with a variety of front-end sample enrichment methods for specific PTMs, bottom-up proteomics gains broad applications for the high-throughput quantitative analysis of proteins.

Intact glycopeptides can be identified based on previously known glycosites and their potential glycan structures. For example, one could identify *N*-glycosites using the previously discussed methods such as SPEG, from which peptides containing glycosites can be created as a glycopeptide database. On the other hand, the released *N*-glycans from glycoproteins could be identified by MS/MS using in-solution methods or GIG to determine their compositions to establish an *N*-glycan database. When a complex protein mixture is directly digested by trypsin, it consists of intact glycopeptides and other non-glycosylated peptides. The intact glycopeptides can be specifically enriched by HILIC (Boersema et al., 2008; Xu et al., 2008). It is worth noting that HILIC isolation may also enrich other peptides containing hydrophilic PTMs, such as phosphopeptides (McNulty and Annan, 2008). A recently developed technique, electrostatic repulsion hydrophilic interaction chromatography (ERHIC), enables the simultaneous characterization of intact glycopeptides and phosphopeptides (Alpert, 2008; Zhang et al., 2010a). The ratio of intact glycopeptides over phosphopeptides using ERHIC instead of HILIC increases from 20 to 80%, yet intact glycopeptides cannot be completely isolated from phosphopeptides.

The MS spectra of intact glycopeptides can be searched against a glycopeptide library using spectral library matching or precursor mass matching. A recently developed software, termed GPQuest, is based on a spectral library matching algorithm for the site-specific assignment of MS/MS spectra to *N*-glycopeptides (Toghi Eshghi et al., 2015) and has been successfully used to analyze glycopeptides in a variety of cancer (Shah et al., 2015; Sun et al., 2015) and cardiac diseases (Yang et al.,

2015a). It requires databases for *N*-glycans, *O*-glycans, deglycosylated *N*-glycopeptides, and deglycosylated *O*-glycopeptides for glycopeptide identification. Another approach, *p*-Glyco, has been developed by combining complementary higher energy collision dissociation (HCD)-MS/MS, collision induced dissociation (CID)-MS/MS, and MS3 spectra (Zeng et al., 2016), enabling the identification of intact *N*-glycopeptides with high confidence.

#### 16.2.4.2 Top-Down Approaches

The strength of top-down proteomics lies in detecting intact proteins in their native states and keeping their PTM information intact. The identification of PTMs is of great significance since PTMs are postulated to be relevant in many physiological and pathological processes. In addition, top-down approaches have simplified sample preparation by eliminating the time-consuming and inconsistent protein digestion. The analysis of intact glycoproteins by MS provides the most direct route for the identification and characterization of these highly complex species. Since intact glycoproteins may potentially contain different glycoforms, it would be beneficial to add the second dimension such as liquid chromatography (LC) separation prior to MS detection to reduce the sample complexity and enhance the ability to decipher intact glycoproteins.

Capillary electrophoresis (CE) is a powerful analytical technique for the separation of charged molecules such as intact proteins (Whitehouse et al., 1989; Haselberg et al., 2007). It separates ions based on their electrophoretic mobility under the influence of an electric field, and it is often performed in a capillary or a microfluidic device that is compatible with an electrospray ionization (ESI) interface (Yang et al., 2009; Liu et al., 2010). CE-ESI-MS has been reported for the separation and analysis of intact prostate-specific antigen (PSA) (Santos et al., 2015). Recently, this technique has gained increasing attention in clinical applications requiring the high reproducibility and comparability of acquired data. For example, CE-ESI-MS has been used to analyze the human urinary proteome for biomarker discovery and disease diagnostics (Coon et al., 2008). It could also represent a promising tool for top-down glycoprotein analysis.

#### 16.2.4.3 MS/MS Fragmentation Methods for Glycopeptides

Intact glycopeptides can be studied by different MS/MS fragmentation methods, including CID, HCD, and electron transfer dissociation (ETD). In CID, parent peptide ions are accelerated by an electrical potential and collide with inert gas, resulting in random bond breakage to

generate fragment ions for peptide sequence determination. HCD is essentially a higher energy form of CID that can generate additional fragment ions to provide more information for sequence assignment. ETD is a method that is particularly suitable for fragmenting multiply charged large parent ions (Dass, 2007). Unlike CID or HCD that fragments the backbone of both peptides and glycans, ETD fragmentation of intact glycopeptides can break the peptide backbone while allowing glycans and the linkage between a glycan and its attached amino acid (Asn for *N*-glycans and Ser/Thr for *O*-glycans) to remain intact, permitting the localization of glycosites and the structural determination of glycans (Wuhrer et al., 2007; Creese and Cooper, 2012). Because CID or HCD can generate fragment ions for both glycans and peptides, analyzing intact glycopeptides often combines MS/MS spectra from CID or HCD with ETD (Singh et al., 2012).

## 16.3 Glycoprotein Biomarker Quantification Using LC-MS

Quantitative proteomics is the global measurement of protein concentrations in complex biological samples (Ong and Mann, 2005), allowing a quantitative comparison of proteins among healthy and diseased patients for biomarker discovery. There are several stable isotope-based and label-free-based methods that are amenable to the quantification of glycoproteins. The unique aspects and limitations of some of the widely used quantitative methods are discussed in this section.

### 16.3.1 Quantification by Stable Isotope Labeling

Stable isotope tags are often used for the relative quantification of peptides in bottom-up approaches. An extensive list of tags that react with different amino acids (Ong and Mann, 2005) has been developed in the past decade. For example, isobaric tags for relative and absolute quantification (iTRAQ) (Ross et al., 2004) or tandem mass tag (TMT) (Thompson et al., 2003) can label primary amines such as the peptide N-terminus and lysine. Methyl or ethyl esterification is used to react with carboxyl groups including the peptide C-terminus, aspartic acid, and glutamic acid (Goodlett et al., 2001; Syka et al., 2004). Isotope-coded affinity tag (ICAT) (Gygi et al., 1999) and HysTag (Olsen et al., 2004) couple with the sulphydryl group on cysteine. The most commonly used stable isotope tags are listed in Table 16.3.

Amine-reactive tags such as iTRAQ, TMT, deuterium isobaric amine-reactive tag (DIART) (Zhang et al., 2010b), isotope-coded protein label (ICPL) (Schmidt et al., 2005), neutron encoding (NeuCode) (Hebert et al., 2013), and

quantitation using enhanced signal tags (QUEST) (Beardsley and Reilly, 2003) surpass the performance of tags targeting other amino acids because every tryptic peptide contains at least one amine group and the reaction between these tags and amines is highly specific. On the basis of how labeled peptides are quantified, these tags can be classified into two categories: mass-shift and isobaric tags. Mass-shift tags like NeuCode introduce a mass difference onto peptides from different samples, and the relative quantification is achieved using MS1-level spectral information. Peptides labeled by isobaric tags, on the other hand, do not show a mass difference at MS1 level, and the quantification is achieved after MS/MS fragmentation. This is due to the unique design of isobaric tags. For example, 4-plex iTRAQ tags are a group of four molecules with an identical chemical structure consisting of a reporter, a balancer, and an amine-reactive *N*-hydroxysuccinimide group (Figure 16.7a). Because they contain different isotopes in different positions, the reporter ions in a set differ in mass by 1 Da. However, the mass difference is compensated by the balancer, so the total mass of the four tags remains the same (Figure 16.7b). When four samples are labeled with iTRAQ, respectively, and pooled together, differentially labeled peptides yield single MS1 peaks with an identical mass. Upon MS/MS fragmentation, the intensities of the newly generated low mass reporter ions represent the relative abundance of the peptides in the original samples (Figure 16.7c). As a result, protein quantification is achieved based on these reporter ions. iTRAQ tags are also available in an 8-plex format.

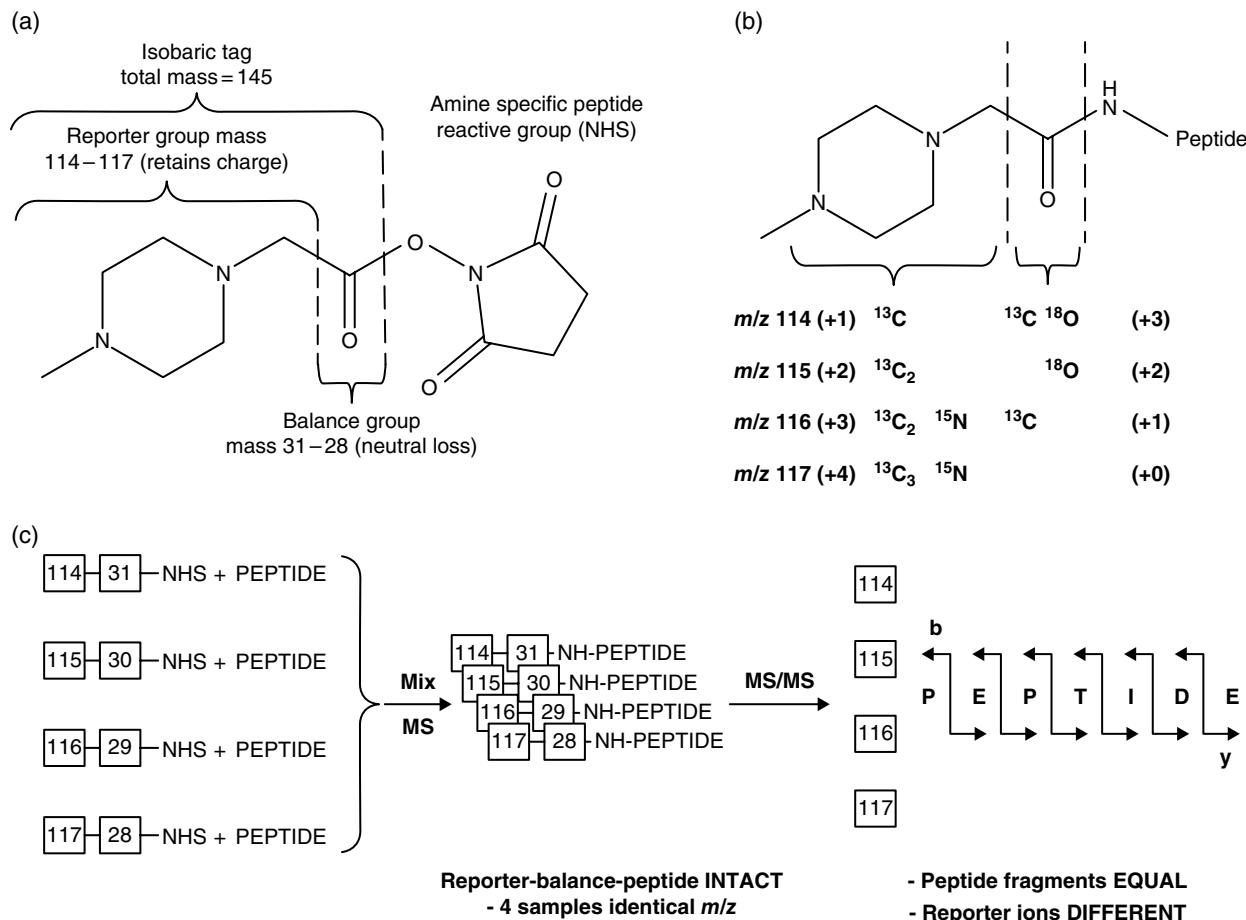
A similar strategy has been employed for the relative quantification of glycans (Hahne et al., 2012; Yang et al., 2013c, 2015b). Unlike peptides, glycans released from glycoproteins usually have an aldehyde at their reducing end, which is suitable for labeling. Aldehyde-reactive groups include primary amine (Yang et al., 2013c, 2015b), hydrazide, and aminoxy (Hahne et al., 2012). AminoxyTMT is commercially available for *N*-glycan isobaric labeling, but it presents a few obstacles for the quantitative analysis of *N*-glycans. It is not straightforward to achieve complete labeling using aminoxyTMT, and multiple adducts can form that interfere with glycan identification. The resulting reporter ions of AminoxyTMT-labeled glycans upon MS/MS fragmentation are also not of sufficient intensity to permit accurate quantification. A novel tag, QUANTITY, overcomes these problems and has been applied for the high-throughput quantitative analysis of *N*-glycans (Yang et al., 2015b).

### 16.3.2 Metabolic Labeling Strategies

Stable isotope labeling with amino acids in cell culture (SILAC) was introduced in 2002 as a method for the quantification of proteins expressed in different cells

**Table 16.3** Stable isotope tags for quantitative analysis of peptides.

Symbol	Stable isotope tag	Reactive group	Targeted group	Quantification method
iTRAQ	Isobaric tags for relative and absolute quantification (Ross et al., 2004)	NHS ester	Amine, lysine, or N-terminal	MS2
TMT	Tandem mass tag (Thompson et al., 2003)	NHS ester	Amine, lysine, or N-terminal	MS2
ICAT	Isotope-coded affinity tag (Gygi et al., 1999)	Iodoacetamide	Cysteine	MS1
DIART	Deuterium isobaric amine-reactive tag (Zhang et al., 2010b)	NHS ester	Amine, lysine, or N-terminal	MS2
ALICE	Acid-labile isotope-coded extractants (Qiu et al., 2002)	NHS ester	Amine, lysine, or N-terminal	MS1
HysTag	Isotopically labeled cysteine-tagging and complexity-reducing reagent (Qiu et al., 2002)	2-Thiopyridyl disulfide	Cysteine	MS1
ICPL	Isotope-coded protein label (Schmidt et al., 2005)	NHS ester	Amine, lysine, or N-terminal	MS1
MCAT	Guanidination ( <i>O</i> -methylisourea) mass-coded abundance tagging (Cagney and Emili, 2002)	<i>O</i> -methylisourea	Lysine	MS2
QUEST	Quantification using enhanced sequence tags (Beardsley and Reilly, 2003)	Thioacetimidate	Lysine	MS1
NBSCI	2-Nitrobenzenesulfenyl chloride (Kuyama et al., 2003)	Nitrobenzenesulfenyl	Cysteine	MS1
PhIAT	Phosphoprotein isotope-coded affinity tag (Goshe et al., 2001)	$\beta$ -elimination, Michael addition	Phosphoseryl, phosphothreonyl	MS1



**Figure 16.7** Multiplexed isobaric tagging for the relative and absolute quantification (iTRAQ) of peptides from multiple biological samples. (a) iTRAQ chemical component: reporter, balancer, and reactive group. (b) Reporter stable isotope codes. (c) Labeling and quantification strategy. Source: Ross et al. (2004) <http://www.mcponline.org/content/3/12/1154.short>. Used under CC BY 4.0 <https://creativecommons.org/licenses/by/4.0/>. Reprinted with permission from ASBMB.

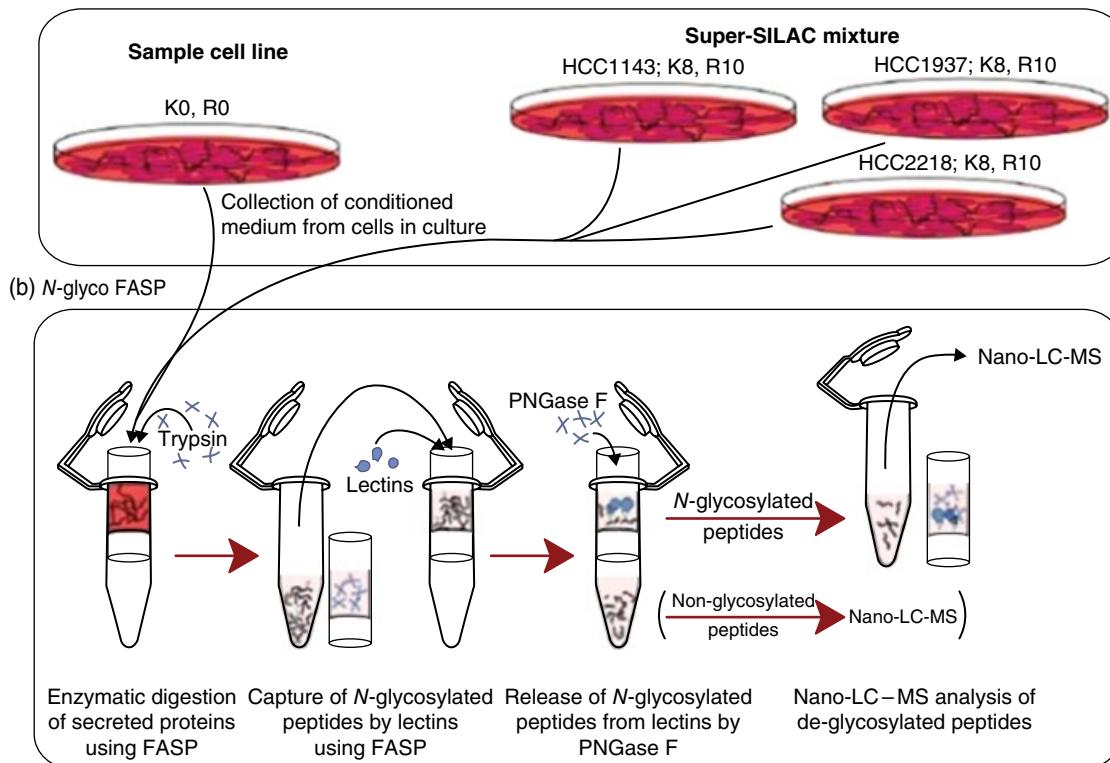
(Ong et al., 2002; Ong and Mann, 2006) (Figure 16.8). Stable isotope-labeled essential amino acids such as  $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$ -Arg and  $^{13}\text{C}_6$ ,  $^{15}\text{N}_2$ -Lys are added into cell culture media lacking these amino acids and are therefore incorporated into all proteins as they are synthesized (Ong et al., 2002). Mixing these cells encoded by heavy amino acids with those encoded by light counterparts enables the quantitative determination of the corresponding proteomes with high accuracy. The technique has been used with an *N*-glyco filter-assisted sample preparation (FASP) protocol to focus on secreted or shed proteins (Boersema et al., 2013). In this study, a super-SILAC mixture including three cancer cell lines encoded by  $^{13}\text{C}_6$ ,  $^{15}\text{N}_4$ -Arg and  $^{13}\text{C}_6$ ,  $^{15}\text{N}_2$ -Lys was mixed with a cancer cell line grown in a medium containing regular Arg and Lys (Figure 16.8a). Secreted proteins were collected and digested by trypsin. *N*-glycosylated peptides were captured by immobilized lectins (Figure 16.8b) and then released by PNGase F treatment for LC-MS/MS analysis.

A validation experiment confirmed that both light- (Figure 16.8c) and heavy-encoded *N*-glycosylated peptides (Figure 16.8d) showed good correlation, which was further improved by normalization of the relative quantification results (Figure 16.8e, f).

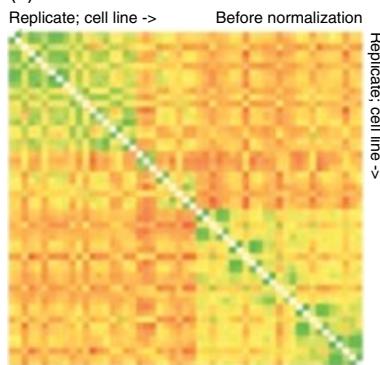
### 16.3.3 Label-Free Glycoprotein Quantification

Several label-free strategies have been employed for the relative quantification of glycans, glycopeptides, and glycoproteins, including spectral counting and MS signal intensity-based quantification. A spectral counting strategy has been developed to enable the site-specific profiling of *N*- and *O*-linked glycosylation on recombinant HIV protein gp120, which permitted the elucidation of various high mannose structures and hybrid/complex glycans (Yang et al., 2014). Spectral counting has also been applied to conduct an in-depth analysis of the *N*-glyco-secretome of human hepatocellular carcinoma metastatic cell lines (Li et al., 2016).

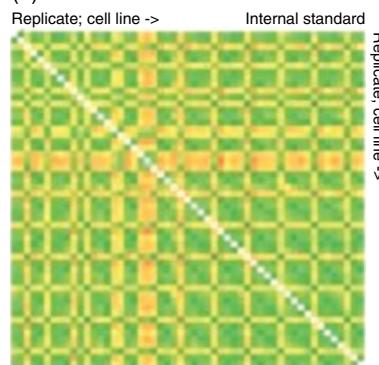
## (a) Cell culture, sample preparation



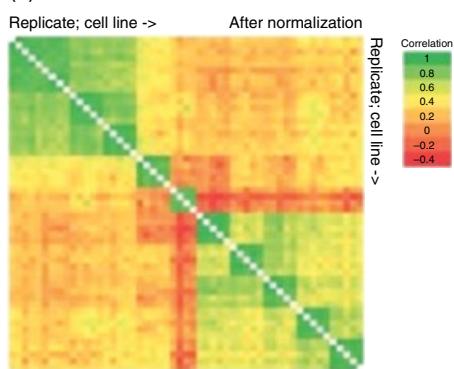
(c)



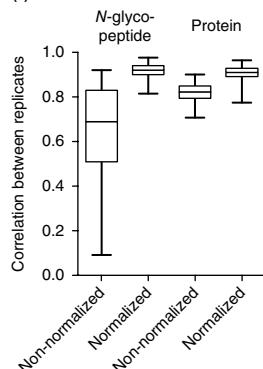
(d)



(e)



(f)



**Figure 16.8** SILAC labeling and glyco-capture for quantitative analysis of secretome. (a) SILAC cell culture. (b) N-glyco-filter-aided sample preparation (FASP). (c-f) Effect of normalization using the super-SILAC internal standard. Source: Boersema et al. (2013). <http://www.mcponline.org/content/12/1/158.short>. Used under CC BY 4.0 <https://creativecommons.org/licenses/by/4.0/>. Reprinted with permission from ASBMB.

Spectral counting is a semiquantitative measurement of protein abundance (Liu et al., 2004; Trudgian et al., 2011). In a discovery proteomics experiment (shotgun experiment), the number of MS/MS events triggered by a single peptide are summed, which corresponds to spectral counts and is in correlation with peptide abundance (Bantscheff et al., 2007). The comparison of the relative abundance of a protein across samples is then achieved by comparing the spectral counts for all peptides associated with a given protein among different samples (Old et al., 2005).

Label-free protein quantification methods based on MS signal intensity generally measure ion abundances at specific retention times for the given ionized peptides. For accurate quantification, it is necessary to process raw LC-MS/MS data to align the retention times across multiple runs in order to achieve comparative assessment of peptides across experiments. The data processing algorithms also optimize peak picking, suppress spectral noise, and normalize peak abundance (Neilson et al., 2011). Software tools available for label-free quantification include Scaffold, emPAI, APEX, Census, Sieve, PEPPeR, MaxQuant, MFPaQ, and Skyline, among others (Ramus et al., 2016). Regardless of which label-free method is chosen, sample preparation, sample loading, LC separation, and MS performance must have a high level of reproducibility to ensure the reliability of the results reported by the respective software tools (Ong and Mann, 2005).

#### 16.3.4 Methods for Targeted Quantification Using LC-MS/MS

The ability to quantify individual glycans is important because changes in their abundance are often associated with significant biological events. However, the presence of glycoforms that differ in branch and/or linkage position renders the identification and quantification of individual glycans rather challenging. Selected reaction monitoring (SRM) MS methodologies have been used successfully with glycan quantification (Goldman and Sanda, 2015).

SRM (or multiple reaction monitoring (MRM)) is a targeted MS approach that has become increasingly popular for protein quantification. In contrast to discovery proteomics experiments, SRM detects multiple pre-selected peptides that serve as surrogates for proteins of interest. These proteins are often potential biomarker candidates identified from discovery proteomics experiments. Usually, SRM assays are performed using a triple-quadrupole mass spectrometer, in which the precursor ions and their characteristic fragment ions are selected in the first and third quadrupole analyzers, respectively, while the second quadrupole serves as the collision cell

for fragmentation. This double-filter mechanism makes it possible to detect targeted peptides with high confidence. Therefore, SRM assays are considered as a gold standard for MS-based protein quantification because of their high specificity, reproducibility, accuracy, and compatibility with multiplex measurement (hundreds of peptides can be quantified in a single assay) (Boja and Rodriguez, 2012; Largy et al., 2013; Ebhardt et al., 2015).

In most glycan studies using SRM, glycan identification is based on diagnostic oxonium ions (Song et al., 2012) or common fragment ions for all glycans (Gil et al., 2009). Recently, SRM has been used to quantify sialylated N-glycan linkage isomers (Tao et al., 2014) and the isomers of unsulfated core 1 O-glycans (Flowers et al., 2013). SRM has also been used for the quantification of N-linked glycopeptides (Thomas et al., 2015).

Parallel reaction monitoring (PRM) is a targeted MS strategy that is similar to SRM but is commonly performed on a quadrupole Orbitrap mass spectrometer or quadrupole TOF mass spectrometer, leveraging the ion-selecting and high resolution capability of the instrument (Peterson et al., 2012; Gallien and Domon, 2015; Bourmaud et al., 2016) to provide high sensitivity and selectivity. PRM has been used to detect differences in the relative abundance of endogenous N-linked glycopeptides in the serum of prostate cancer patients (Kim et al., 2012).

### 16.4 Protein Biomarkers for Clinical Applications

Disease-specific biomarkers have great value for the early detection of diseases and the selection of proper therapies. Quantitative MS methods have become increasingly integrated with biomarker discovery and validation. However, the translation of biomarkers discovered in basic research to clinical applications will be possible only after strict preclinical and clinical criteria have been met (Crutchfield et al., 2016). Here, we provide an overview of the various types of biomarkers.

#### 16.4.1 FDA-Approved Glycoprotein Biomarkers

Many protein biomarkers have been approved by the FDA for clinical applications and have been reviewed extensively in the literature (Polanski and Anderson, 2006; Füzéry et al., 2013; Yotsukura and Mamitsuka, 2015). Table 16.4 provides a list of 28 FDA-approved protein biomarkers that are often used for clinical diagnosis, prognosis, or monitoring disease response to therapy, most of which are glycoproteins. As shown in Table 16.4, 4 out of 28 biomarkers are non-glycosylated proteins, including ESR1 (estrogen receptor), PGR (progesterone

receptor), TP63 (p63 protein), and NUMA1 (nuclear mitotic apparatus protein). However, ESR1 may be *O*-glycosylated by O-GlcNAc in signal transduction regulation (Wells et al., 2001) and NUMA1 has one potential *O*-glycosite (Wang et al., 2010). P63, a biomarker that is not glycosylated but rather ubiquitinated, has been utilized for the differential diagnosis of prostate cancer from formalin-fixed, paraffin-embedded (FFPE) tissue (Li and Xiao, 2014). The only other non-glycosylated biomarker is PGR, yet it is heavily modified by phosphorylation.

Interestingly, the other 24 disease biomarkers listed in Table 16.4 are either *N*-glycosylated, *O*-glycosylated, or both. Among them, 14 are *N*-glycosylated proteins, 3 are *O*-glycosylated, and 7 are modified by both *N*-glycans and *O*-glycans. Thirteen proteins are also modified by phosphorylation. Changes of these PTMs often contribute to tumorigenesis or metastasis. For example, the glycosylation of ORM1 is increased in ovarian cancer serum compared to normal serum (Saldova et al., 2008). The fucosylation of  $\alpha$ -fetoprotein often occurs on *N*-glycans, *O*-glycans, or glycolipids, which is very important for Notch signaling and has been reported in several pathological conditions, especially in hepatocellular carcinomas (Sato et al., 1993).

The 28 FDA-approved protein biomarkers are grouped according to their disease relevance. There are six different breast cancer biomarkers, including AGP, CA15-3, CA27.29, ESR1 Her-2/neu, and PGR. These biomarkers have different sensitivity and specificity as shown in Table 16.4. Four biomarkers are specific for ovarian cancer, including apolipoprotein A-1 (APOA1), CA125 (MUC15), transthyretin (TTR), and HE4 (WFDC2), while four biomarkers are available for prostate cancers, namely, free PSA (KLK3), p63, pro2PSA, and total PSA. Two general pan-cancer biomarkers are TGF $\beta$  and  $\beta$ -2-microglobulin (B2M).

A panel of glycoproteins has been discovered as potential biomarkers for pancreatic cancer by quantitative proteomics analysis (Nie et al., 2014). In order to predict the malignancy of ovarian cancer, the immunoassay-based OVA1 test is required to measure a panel of biomarkers such as CA125, prealbumin (TTR), APOA1, B2M, and TFRC (Fung, 2010). Indeed, this OVA1 test improves the confidence of presurgical adnexal mass management and helps to detect more ovarian cancer than standard testing (Ueland et al., 2010). Another example of a diagnostic test based on ovarian cancer biomarkers is the Risk of Ovarian Malignancy Algorithm (ROMA) assay, in which two biomarkers are tested, including HE4 and CA125 (Van Gorp et al., 2011).

Although the majority of FDA-approved biomarkers are glycoproteins, the analytical platforms that were utilized for their discovery and validation consisted predominantly of immunoassays. Hence, many of these

assays lack the requisite specificity to adequately determine the heterogeneity of the glycan structures that are attached to the proteins of interest, which creates potential challenges in identifying the specific relevance of glycosylation in the context of these biomarkers. However, glycan structure information can be gleaned from other analytical methods such as MS, as previously described in Sections 16.2.4 and 16.3. Given the specificity and selectivity of MS-based analytical approaches, it is possible to design studies to determine whether observed glycoprotein changes are due to differences in abundance at the protein or glycan level to consequently improve the diagnostic performance of certain glycoprotein biomarkers.

#### 16.4.2 Classes of Biomarkers

Diagnostic biomarkers aid disease diagnosis. They are commonly used as measurable indicators of the presence of a disease state. A prognostic factor is a clinical or biologic trait that is objectively measurable and provides information on the outcome of disease in an untreated individual (Italiano, 2011). As listed in Table 16.4, protein biomarkers such as BNP (NPPB), CA125 (MUC16), c-kit (KIT), estrogen receptor (ESR1), haptoglobin (HP), NMP22 (NUMA1), TGF $\beta$ , total PSA (KLK3), and cardiac troponin I (TNNI3) can be used for disease diagnosis. For example, BNP has been employed in the emergency diagnosis of heart failure (McCullough et al., 2002), as well as in the diagnosis of congestive heart failure in an urgent care setting (Dao et al., 2001).

A predictive biomarker is a particular protein that indicates sensitivity or resistance to a specific therapy. The use of predictive markers has been increasingly popular in cancer therapy as it allows for the improved identification of patients who would respond positively to the therapy. AGP, APOA1, CA125, TFRC (transferrin), TTR, and B2M belong to this category.

#### 16.4.3 New Glycoprotein Biomarker Discovery

Because many diseases still lack reliable biomarkers, considerable effort has been devoted to identifying novel biomarkers that can improve the accuracy of diagnosis and detect diseases as early as possible to increase patient survival rate. A variety of strategies have been pursued to meet these goals. Figure 16.9 summarizes one biomarker discovery strategy. The flowchart schematically sketches how to discover the potential biomarkers, validate the candidates, and implement them. Even though this strategy is proposed for the study of biomarkers from extracellular vesicles (Nawaz et al., 2014), it may be useful for the discovery of glycoprotein biomarkers. Briefly, proteins are extracted from patients' body fluids, from which glycoproteins are specifically enriched by SPEG

**Table 16.4** FDA-approved protein biomarkers for clinical application.

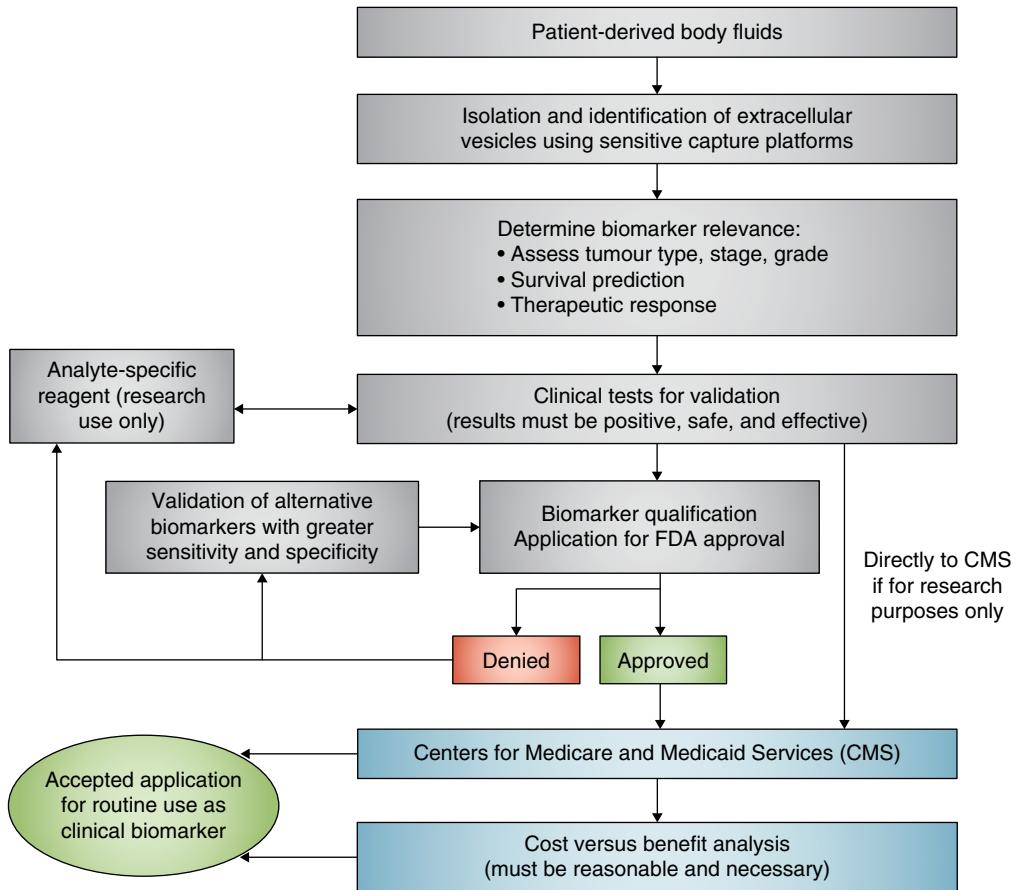
Biomarker	Gene	Disease type	PTM	Clinical application	Specimen	Method	Location	Type	Cutoff	Sensitivity (%)	Specificity (%)	Approval year
α-1-Acid glycoprotein (AGP) (Barroso-Sousa et al., 2013)	ORM1	Breast	N-glycosylation	Predictive	Peripheral blood	Immunoassay	Extracellular space	Other	1.2 mg/mL	45.5	88.1	2009
α-Fetoprotein (AFP) (Zinkin et al., 2008)	AFP	Hepatocellular carcinomas	N-glycosylation, phosphorylation	Prognostic	Serum	HPLC, immunoassay	Extracellular space	Transporter	20 ng/mL	50	70	1992
Apolipoprotein (A-1) (Moore et al., 2006; Nolen and Lokshin, 2013)	APOA1	Ovarian	N-glycosylation, O-glycosylation, phosphorylation	Predictive	Peripheral blood	Immunoassay	Extracellular space	Transporter	NA	52.4	96.5	2009
B-type natriuretic peptide (BNP) (Dao et al., 2001)	NPPB	Heart	O-glycosylation	Diagnostic	Blood	Immunoassay	Extracellular space	Other	8 pg/mL	98	92	2000
Cancer antigen 125 (CA125) (Dabrowska et al., 2004; Stieber et al., 2006)	MUC16	Ovarian	N-glycosylation, O-glycosylation, phosphorylation	Predictive, prognostic	Serum	Immunoassay	Cytoplasm	Other	95 IU/mL	84	80	1997
Cancer antigen 15-3 (CA15-3) (Skates et al., 2004)	MUC1	Breast	N-glycosylation, O-glycosylation, phosphorylation	Monitoring disease response to therapy	Serum, plasma	Immunoassay	Plasma membrane	Other	40 U/mL	58.2	96	1997
Cancer antigen 19-9 (CA19-9) (Tomonaga et al., 2004)	MUC1	Pancreatic	N-glycosylation, O-glycosylation, phosphorylation	Monitoring disease status	Serum, plasma	Immunoassay	Plasma membrane	Other	NA	75	80	2002
Cancer antigen 27.29 (CA27.29) (Chan et al., 1997)	MUC1	Breast	N-glycosylation, O-glycosylation, phosphorylation	Monitoring disease response to therapy	Serum	Immunoassay	Plasma membrane	Other	39 U/mL	60	93	2002
Carcinoembryonic antigen (CEA) (Yamamoto et al., 2004)	CEACAM5	Peritoneal cancer dissemination	N-glycosylation	Prognosis	Serum, plasma	Immunoassay	Plasma membrane	Other	0.5 ng/mL	75.8	90.8	1985
c-Kit (CD117) (Hoehn et al., 2012)	KIT	Gastrointestinal stromal tumors	N-glycosylation, phosphorylation	Diagnostic	FFPE tissue	Immunohistochemistry	Plasma membrane	Transmembrane receptor	NA	100	94	2004
DR-70 (fibrinogen degradation product) (Yesil et al., 2013)	FGA	Colorectal	N-glycosylation, O-glycosylation, phosphorylation	Monitoring progression of disease	Serum	Immunoassay	Extracellular space	Other	0.7 µg/mL	53.9	96	2008

(Continued)

**Table 16.4** (Continued)

Biomarker	Gene	Disease type	PTM	Clinical application	Specimen	Method	Location	Type	Cutoff	Sensitivity (%)	Specificity (%)	Approval year
Estrogen receptor (ER) (Chen et al., 2012)	ESR1	Breast	O-glycosylation, phosphorylation	Prognosis, response to therapy	FFPE tissue	Immunohistochemistry	Nucleus	Ligand-dependent nuclear receptor	NA	97	79	1999
Free PSA (Catalona et al., 2000; Chen et al., 2012)	KLK3	Prostate	N-glycosylation	Discriminating cancer from benign disease	Serum	Immunoassay	Extracellular space	Peptidase	4 ng/mL	90	29	1997
Haptoglobin (Ahn and Cho, 2013)	HP	NSCLC	N-glycosylation	Diagnostic, prognostic, predictive	Blood plasma	Immunoassay	Extracellular space	Peptidase	NA	82.6	63.2	NA
Human epididymis protein 4 (HE4) (Van Gorp et al., 2011)	WFDC2	Ovarian	N-glycosylation	Monitoring recurrence or progression of disease	Serum	Immunoassay	Extracellular space	Other	70 U/mL	84.5	83.3	2008
Hemoglobin (Karl et al., 2008)	HBB	Colorectal	N-glycosylation, phosphorylation, acetylation	Detection of fecal occult blood (home use)	Feces	Lateral flow immunoassay	Cytoplasm	Transporter	20 µg/g (feces)	83	95	NA
Her-2/neu (Cook et al., 2000)	ERBB2	Breast	N-glycosylation, phosphorylation	Assessment for therapy	FFPE tissue	Immunohistochemistry	Plasma membrane	Kinase	15 ng/mL	40	98	1998
Nuclear mitotic apparatus protein (NuMA, NMP22) (Grossman et al., 2006)	NUMA1	Bladder	O-glycosylation, phosphorylation, acetylation	Diagnosis and monitoring of disease (professional and home use)	Urine	Sandwich immunoassay	Nucleus	Other	10 U/mL	49.5	87.3	1996
p63 protein (Ng et al., 2007)	TP63	Prostate	No PTM	Aid in differential diagnosis	FFPE tissue	Immunohistochemistry	Nucleus	Transcription regulator		97.3	95.5	2005
Pro2PSA (prostate-specific antigen) (Lazzeri et al., 2013)	KLK3	Prostate	N-glycosylation	Discriminating cancer from benign disease	Serum	Immunoassay	Extracellular space	Peptidase	60%	96.6	90.9	2012
Progesterone receptor (PR) (Dekker et al., 2015)	PGR	Breast	Phosphorylation	Prognosis, response to therapy	FFPE tissue	Immunohistochemistry	Nucleus	Ligand-dependent nuclear receptor	1%	94.8	92.6	1999
TGFβ (Malaguarnera et al., 2010)	TGFB	Malignant tumor	N-glycosylation	Diagnostic, prognostic	Urine	ELISA	Extracellular space	Growth factor	NA	NA	NA	NA

Thyroglobulin (Lima et al., 2002)	TG	Thyroid	N-glycosylation	Aid in monitoring	Serum, plasma	Immunoassay	Extracellular space	Other	2.3 ng/mL	74.5	95	1997
Total PSA (Loeb et al., 2011)	KLK3	Prostate	N-glycosylation	Prostate cancer diagnosis and monitoring	Serum	Immunoassay	Extracellular space	Peptidase	4.0 ng/mL	46	91	1986
Transferrin (Dowlati et al., 1997)	TFRC	Cancer	N-glycosylation, O-glycosylation, phosphorylation	Predictive	Peripheral blood	Immunoassay	Plasma membrane	Transporter	0.75 µg/mL	91	59	2009
Transthyretin (TTR/ prealbumin) (Rein et al., 2011)	TTR	Ovarian	N-glycosylation, phosphorylation	Predictive	Peripheral blood	Immunoassay	Extracellular space	Transporter	2 mg/mL	76.5	63.6	2009
Troponin I (Ross et al., 2000; Keller et al., 2009)	TNNI3	Cardiac	Phosphorylation, acetylation	Diagnostic	Tissue	ECG	Cytoplasm	Transporter	0.6 ng/mL	94	81	1998
β2-microglobulin (Toth et al., 2013; Yoo et al., 2014)	B2M	Cancer	N-glycosylation	Predictive	Blood	Immunoassay	Plasma membrane	Transmembrane receptor	2.5 µg/mL	49.3	52.1	2015



**Figure 16.9** Multistep validation of biomarkers from patient-derived body fluids. The flowchart shows a step-by-step process by which the profiling and discovery of targeted proteins could ultimately be translated into a clinically applicable biomarker signature. At each step defined goals and criteria must be met in order to proceed to the next level. Source: Nawaz et al. (2014). Reproduced with permission of Nature Publishing Group.

or lectin affinity chromatography. The potential biomarkers will be determined based on their relevance to the specific diseases, such as assessment of tumor type, stage, and grade or whether these biomarkers can be used for survival prediction (prognostic). Some biomarkers may be used for the indication of therapeutic responses. More importantly, clinical tests will be performed to validate those potential biomarkers to confirm that they will generate results that are positive, safe, and effective (Scatena, 2015). The proposed biomarker will be subjected to FDA approval. If it is denied, the alternative biomarkers with greater sensitivity and specificity should be proposed and submitted for validation. Various tests using these approved biomarkers can then be submitted for approval to healthcare agencies, such as the Centers for Medicare and Medicaid Services (CMS). The biomarkers will then be evaluated using a cost versus benefit analysis.

## 16.5 Summary and Future Direction

Because glycosylation is one of the most common PTMs with critical biological and physiological roles, it is not surprising that the majority of the protein biomarkers that have been approved by the FDA for clinical applications are glycosylated. These biomarkers are used for clinical diagnosis, prognosis, or monitoring disease response to therapy. As many diseases still lack sensitive and specific biomarkers, the analysis of glycosylated proteins presents new opportunities for novel insights into measurable disease states.

The analysis of protein glycosylation is very complex due to the diversity of the types of glycosylation (N-, O-, c- and phosphoglycosylation) and the levels of analysis: glycopeptide, glycoprotein, and glycan. Several glycosylation enrichment methods have been developed

including chemoenzymatic methods, solid-phase enrichment, lectin affinity approaches, and hybrid chemical metabolic labeling methods. Equally as important to the deployment of these enrichment approaches are the development of analytical methods, many of which entail the use of various MS technologies. These technologies have facilitated the top-down analysis of intact glycoproteins, the bottom-up analysis of glycopeptides resulting from the enzymatic digestion of glycoproteins, and the analysis of glycans. MS technologies have not only enabled qualitative glycosylation analysis but also made possible quantitative analysis. There are many label-free and stable isotope tag labeling-based quantitative MS methods. The most prevalent non-labeling methods include SRM, spectral counting, and MS signal intensity measurement, whereas the most commonly used chemical and metabolic labeling strategies for quantitative glycosylation analysis include iTRAQ, TMT, ICAT, HysTag, DIART,

ICPL, QUEST, QUANTITY, and SILAC. Software development has also played an integral role in advancing glycoprotein analysis.

There is a great potential for the continuing development of glycoproteins as disease markers to improve the accuracy of diagnosis and to facilitate early disease detection. However, the diverse nature of glycosylation presents unique technical challenges for the development of candidate glycoprotein biomarkers with adequate sensitivity and specificity. Technologies such as LC-MS that facilitate the detailed analysis of glycoforms differing in branching and/or linkage position to permit their identification and quantitation have demonstrated great potential in this area. As glycoprotein enrichment strategies, sample processing methods, and analytical techniques continue to be refined (Zinkin et al., 2008), the important roles of glycobiology in human health and disease will become more precisely elucidated.

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## 17

### Targeted Lipid Biomarker Quantitation Using Liquid Chromatography–Mass Spectrometry (LC–MS)

Ashkan Salamatipour<sup>1</sup>, Ian A. Blair<sup>2</sup>, and Clementina Mesaros<sup>1</sup>

<sup>1</sup> Penn SRP and Center for Excellence in Environmental Toxicology, Department of Systems Pharmacology and Translational Therapeutics, University of Pennsylvania, Philadelphia, PA, USA

<sup>2</sup> Center of Excellence in Environmental Toxicology and Penn SRP Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

#### 17.1 Introduction of Lipids

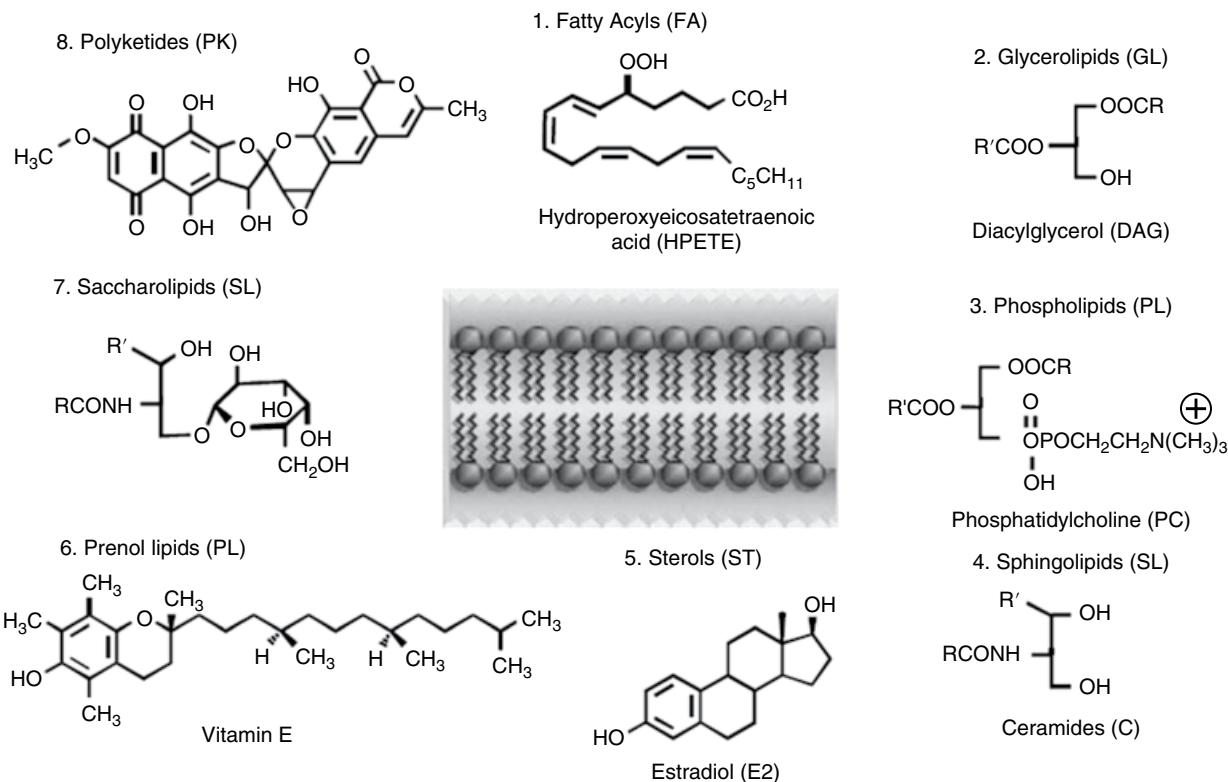
Lipids are a large family of biologically derived molecules that can be divided into eight different classes (Figure 17.1) (Fahy et al., 2005). They have a wide range in polarity, although most lipids are sparingly soluble in water at neutral pH. The first four classes (Figure 17.1) have a very similar biochemistry because of the presence of similar fatty acid (FA) substituents.

Lipids are important components of eukaryotic cell membranes and, in their unmodified forms or after oxidative modification, they are involved in cellular signaling. Phospholipids and glycolipids have a hydrophilic head and hydrophobic tails of long-chain FAs. This facilitates their self-assembly into membranes and lipid droplets, enabling them to coordinate membrane events such as signal transduction (van Meer, 2005). In addition, some lipids (such as sphingosine-1-phosphate and phosphoinositides) participate themselves as potent receptor-mediated inducers of cellular signal transduction pathways (Hannun and Obeid, 2008). Not all the lipids move spontaneously across or between membranes so that their intracellular concentrations are dependent on various transporters that can facilitate translocation of individual lipids across the plasma membrane as well as across membranes of organelles within the cell. It is increasingly being recognized that lipids are playing important roles in all the major human diseases. Cancer (Blair, 2008) and neurodegenerative disorders (Han, 2007) are now considered to have a lipid component. The core of plasma lipoproteins together with the core of lipid droplets in the cytosol is made up of the class 2 lipid triacylglycerol, together with the class 5 lipid cholesterol, which is esterified into various class 1 fatty acyls (Figure 17.1) (van Meer, 2005). In contrast, the bulk of cellular lipids is organized into cellular

membranes. Phospholipases can cleave FAs from the *sn*-2 position of phospholipids to yield class 1 fatty acyls together with the corresponding lysophospholipids. The class 3 (Figure 17.1) lipid phosphatidylinositol acts as a scaffold for the formation of phosphoinositides, which are phosphorylated lipids with potent cell signaling properties.

Sphingomyelin (a class 4 lipid), which is also found in lipid membranes, contains a phosphocholine head group similar to phosphatidylcholine, but it has a hydrophobic ceramide backbone consisting of a sphingosine tail and one saturated FA (Hannun and Obeid, 2008). In glycosphingolipids, ceramide carries carbohydrates, the simplest ones being glucosyl- and galactosylceramide. Breakdown of sphingolipids proceeds through the action of specific hydrolases, which leads to the formation of glucosylceramide and galactosylceramide. In turn, specific  $\beta$ -glucosidases and galactosidases hydrolyze these lipids to regenerate ceramide. Ceramides may be broken down by one of many ceramidases, resulting in the formation of sphingosine, which can be recycled into sphingolipid pathways or phosphorylated by one of two sphingosine kinases to give sphingosine-1-phosphate. Sphingolipids have roles in the regulation of cell growth, death, senescence, adhesion, migration, inflammation, angiogenesis, and intracellular trafficking (Hannun and Obeid, 2008). Elucidating the mechanisms of these varied cell functions makes it necessary to have efficient mass spectrometry (MS) methodology for the analysis of sphingolipids and their metabolites (Levery, 2005). A particularly good method was described by Basit et al. (2015).

The best known representative of the sterol lipids (class 5, Figure 17.1) is the cholesterol, which is an important factor in the etiology of cardiovascular disease (CVD). MS-based methodology has been at the forefront



**Figure 17.1** Structures of the eight lipid classes. The center of the figure shows the structure of a lipid membrane.

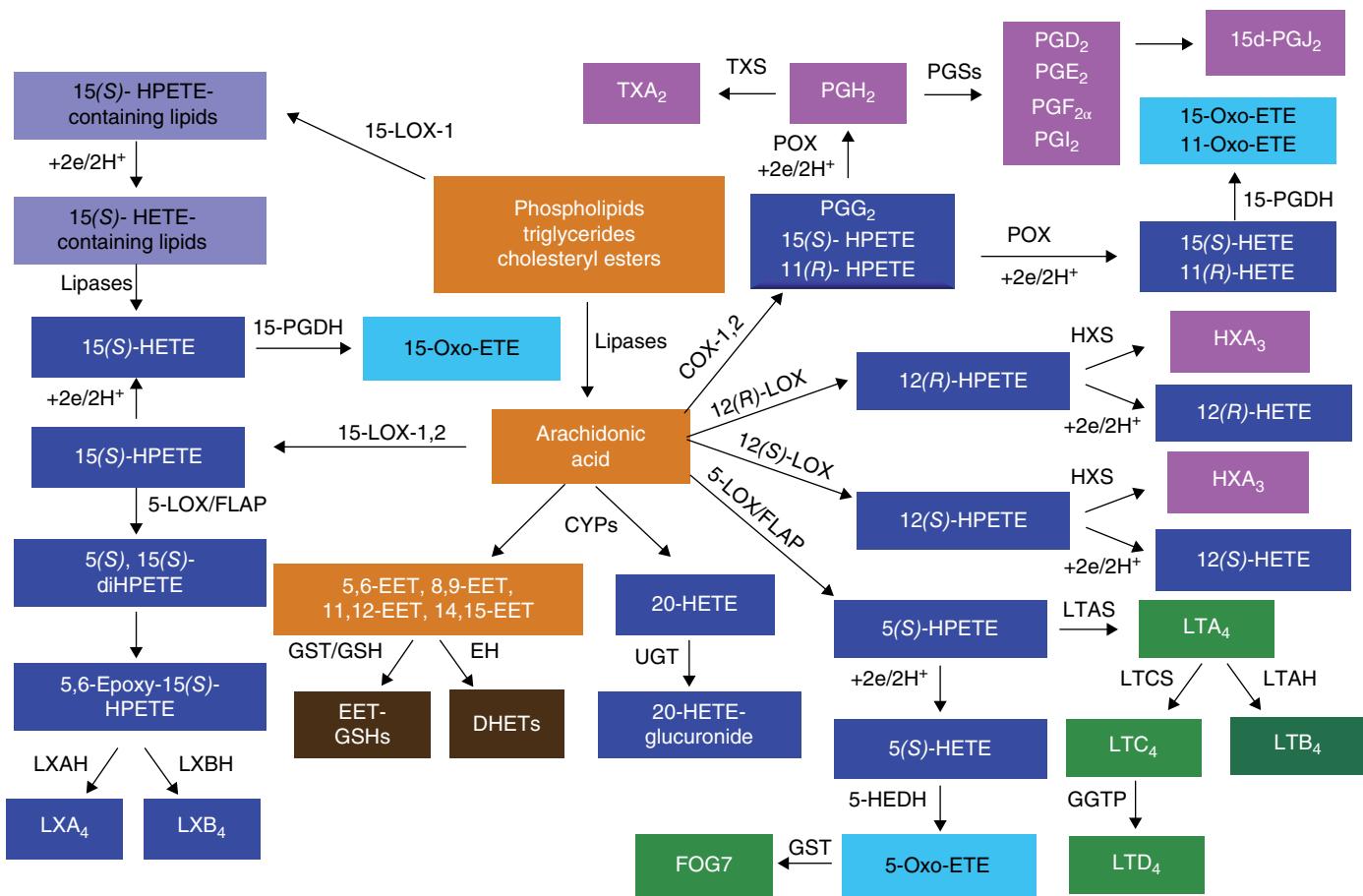
of analyzing sterol lipids (Karu et al., 2007), including cholesterol and its active metabolites (Murphy and Johnson, 2008). Other representatives of class 5 are the estrogen derivatives that usually require high sensitivity in liquid chromatography–mass spectrometry (LC–MS) methods like the ones described by Wang et al. (2015).

Since the first four classes share a similarity in their biochemistry due to the presence of the fatty acyl component, we will focus on the fatty acyls that can suffer oxidative modification by the action of reactive oxygen species (ROS), or various oxygenases.

Arachidonic acid (AA) is one of the most abundant fatty acyl components of the lipidome (<http://www.lipidmaps.org/>). It is esterified in sterol lipids and at the *sn*-2 position of glycerolipids and glycerophospholipids (Chen et al., 2008; Stafforini et al., 2006) (Figure 17.1). It is released by lipases (Taylor et al., 2008) and metabolized to bioactive eicosanoids by cyclooxygenases (COXs), also known as prostaglandin–endoperoxide synthase (PTGS), and lipoxygenases (LOXs) (Funk, 2001; Mesaros et al., 2009) (Figure 17.2). The eicosanoids (from the Greek eikosa, meaning 20) are a large family of AA oxidation products that contain 20 carbon atoms, and many of the eicosanoids are potent lipid mediators of biological processes (Shimizu, 2009). The two isozymes of COXs found in humans are frequently called COX-1 and COX-2 in the medical literature. Selectivity for

COX-2 is the main feature of celecoxib, etoricoxib, and other members of newer nonsteroidal anti-inflammatory drugs (NSAIDs). Because COX-2 is usually specific to inflamed tissue, there is much less gastric irritation associated with COX-2 inhibitors, with a decreased risk of peptic ulceration.

COX-2 can also metabolize AA present in monoglycerides to esterified eicosanoids (Rouzer and Marnett, 2008), whereas 12-LOXs and 15-LOXs can metabolize AA present in glycerolipids, glycerophospholipids, sterol lipids, and complex lipid–protein assemblies to esterified hydroperoxyeicosatetraenoic acids (HPETEs) (Funk and Cyrus, 2001; Kuhn and O'Donnell, 2006; Maskrey et al., 2007). Cytochrome P450 (CYP450) can metabolize free and esterified AA to epoxyeicosatrienoic acids (EETs) (Campbell and Falck, 2007; Chen et al., 2008; Theken and Lee, 2007). ROS can convert both free and esterified AA through nonenzymatic processes into isoprostanes (isopPs) and HPETEs (Moreno, 2009; Vincent et al., 2008). HPETEs formed enzymatically or nonenzymatically on esterified lipids are reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs) by peroxiredoxin VI (PrxVI) (Manevich and Fisher, 2005) or phospholipid hydroperoxide glutathione peroxidase (GPx4) (Kuhn and Borchert, 2002). They are subsequently released by lipases as the corresponding free non-esterified HETEs (Chaitidis et al., 1998). HPETEs formed from



**Figure 17.2** Pathways of AA metabolism. Source: Reprinted with permission from Mesaros and Blair (2012). Abbreviations: COX, cyclooxygenase; CYP450, cytochrome P450; EET, epoxyeicosatrienoic acid; EH, epoxide hydrolase; FLAP, 5-lipoxygenase activating protein; GGT,  $\gamma$ -glutamyltranspeptidase; GSH, glutathione; GST, glutathione-S-transferase; H, hydrolase; HEDH, hydroxyeicosanoid dehydrogenase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HX, hepxillin; LOX, lipoxygenase; LT, leukotriene; LX, lipoxins; PG, prostaglandin; PGDH, prostaglandin dehydrogenase; POX, peroxidase; S, synthase; TX thromboxane; UGT, UDP-glucuronosyltransferases.

free AA are reduced to HETEs by glutathione transferases and peroxidases (Chaitidis et al., 1998; Kuhn and Borchert, 2002; Zhao et al., 1999). The resulting free HETEs from both enzymatic and nonenzymatic pathways can then be converted to oxo-eicosatetraenoic acids (oxo-ETEs), which form glutathione (GSH) adducts (Lee et al., 2007). Alternatively, esterified and free HPETEs undergo homolytic decomposition into highly reactive bifunctional electrophiles, which damage cellular DNA (Blair, 2008; Jian et al., 2005; Lee et al., 2005a, 2005c), RNA (Zhu et al., 2006), proteins, and peptides (Oe et al., 2003; Yocum et al., 2005). Chiral lipidomics analysis of the free and esterified HETEs can provide insight into the structures of lipid precursors that cause this damage, helping to elucidate the roles that lipids play in oxidative stress (Williams et al., 2005).

Prostaglandins (PGs) and thromboxanes (TXs) are COX-derived eicosanoids that have important roles as autacoids involved in the regulation of cardiovascular function (Iniguez et al., 2008) and tumor progression (Wang et al., 2007). Leukotrienes (LTs) are LOX-derived products and have been implicated as important mediators of inflammation, asthma, CVD, and cancer (Peters-Golden and Henderson, 2007). CYP450-derived EETs and their hydration products, the dihydroxyeicosatrienoic acids (DHETs), are vasodilators (Natarajan and Reddy, 2003), whereas CYP450-derived 20-HETE is a vasoconstrictor (Miyata and Roman, 2005). Some ROS-derived isoPs have been implicated as mediators of vasoconstriction in different vascular beds, as smooth muscle cell mitogens, and as mediators of monocyte adhesion to endothelial cells (Cracowski and Durand, 2006; Milne et al., 2008). However, the intense interest in isoPs stems primarily from their utility as biomarkers of oxidative stress (Milne et al., 2008; Pratico et al., 2004).

Free radical-mediated lipid hydroperoxide formation using AA as a substrate can lead to the formation of 24 different HPETE isomers without stereoselectivity. In comparison, COX- and LOX-mediated HPETE formation is highly stereoselective. For example, COX-2 converts AA to 11(R)-, 15(S)-, and 15(R)-HPETE (Lee et al., 2007), while 15-LOX-1 converts it to 12(S)- and 15(S)-HPETE (Funk, 2001; Mesaros and Blair, 2012) (Figure 17.2). In order to distinguish between the enantiomers that are formed by different enzymatic and nonenzymatic pathways, targeted chiral methods are advantageous, leading to elucidation of the metabolism of AA *in vitro* or *in vivo*.

## 17.2 LC-MS Analysis of Lipids

LC-MS is the method of choice for lipid analysis. There is a wide range of the lipids that can be analyzed by MS with the atmospheric pressure ionization (API)-based

techniques of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), together with matrix-assisted laser desorption/ionization (MALDI) (Fenn et al., 1989; Schiller et al., 2007; Siuzdak, 1994). The use of these techniques in combination with collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS) has made it possible to gain important insight into the structures of the lipids and their biological ac LC techniques.

Peterson and Cummings (2006) provided insight into the chromatographic methods used for the analysis of phospholipids in biological samples. Most of the lipid classes could be measured by slight modifications of the methods described there. When choosing an LC method for lipids analysis, most groups choose the use of reversed-phase methodology. The stationary phase is usually a C18 polymer (Duflot et al., 2016; Nalesto et al., 2011), but C8 and C4 columns are also useful in combination with polar mobile phases. Most reported studies have employed robust gradient elution methodology that was readily automated. Normal phase LC, which has been used primarily for the analysis of fatty acyl lipids, employs silica-gel-based columns. The mobile phases are usually nonpolar, hexanes or heptanes, but the polar component can vary widely. Chiral cellulose-coated silica gel columns in combination with nonpolar mobile phase solvents have proved to be extremely useful for the identification of the origin of different bioactive fatty acyl lipids.

The present chapter will focus on targeted LC-MS methods for measuring class I lipids—the fatty acyls in biological samples. Fatty acyls are biologically important lipids, have important roles as autacoids involved in the regulation of cardiovascular function (Iniguez et al., 2008) and tumor progression (Wang et al., 2007), and are important mediators of inflammation, asthma, CVD, and cancer (Peters-Golden and Henderson, 2007). In a 2009 review article (Mesaros et al., 2009), it was noted that there were few reports of targeted approaches for more than one class of eicosanoid. Since that time a number of targeted approaches have appeared. The present chapter will include publications newer than 2009.

Many of the eicosanoids are present in low concentrations with remarkable structural complexity in biological fluids in the presence of other highly abundant lipids, making their analysis particularly challenging (Lee et al., 2005b). The methodology for analyzing eicosanoids is required to be capable of measuring enantiomers and diastereomers with high sensitivity and specificity. The highest sensitivity that can be achieved for the analysis of eicosanoids involves the use of liquid chromatography—multiple reaction monitoring (MRM)/tandem mass spectrometry (LC-MRM/MS/MS). The highest specificity is obtained when the MRM transition is between an intense parent ion that contains the intact molecule (M)

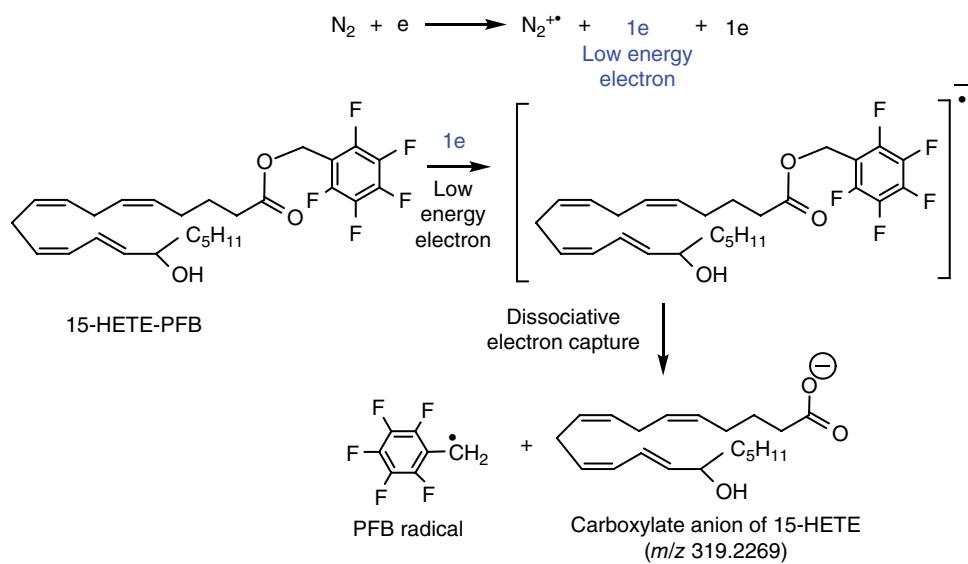
and a structurally significant product ion diagnostic of particular regioisomer. An example of this useful situation arises with HETEs, where product ions are formed through an  $\alpha$ -cleavage adjacent to a double bond. In some cases, fragmentation produced in the collision cell is not very specific, and isomeric eicosanoids sometimes produce similar product ion profiles. An example of this less desirable situation arises with prostaglandin E2 (PGE<sub>2</sub>) and prostaglandin D2 (PGD<sub>2</sub>), where the isomers need to be well separated by LC for correct quantification. Most LC-MRM/MS/MS methods that have been reported employ ESI in the negative ion mode, in which the parent ion arises from de-protonation of the eicosanoid molecule (M) to give an ion corresponding to [M-H]<sup>-</sup>.

To improve the sensitivity for lipid analysis by LC–MS/MS, derivatization approaches have been used, such as in certain cases where derivatization coupled with fluorescence detection is favorable (Yue et al., 2004). Care has to be taken to ensure that uniform derivatization occurs with minimal degradation and that no interfering substances are introduced by the derivatization process and to carefully optimize the chromatographic conditions since frequently the derivation reagents are also the leaving groups in MRM, leading to a less selective product ion. Sometime, improved selectivity can be found by using a MS fragmentation pattern on the backbone of the analyte even that MRM transition may not have the highest intensity. A special case of increased sensitivity in the negative ion mode of APCI occurs with electron capture atmospheric pressure chemical ionization (ECAPCI), which has been applied for analysis of trace amount of lipids in biological samples. The mechanism of ECAPCI is illustrated in Figure 17.3

using the pentafluorobenzyl (PFB)-ester derivative of 15-hydroxyeicosatetraenoic acid (15-HETE) as the model molecule. The low-energy electrons generated in the API source (through interaction of the corona discharge with the nitrogen nebulizing gas) can be captured with a suitable electron-capturing group (such as PFB esters). The initially formed radical anion dissociates (through dissociative electron capture) into a carboxylate anion (Singh et al., 2000) for highly sensitive detection in negative mode. For nonpolar lipids, high sensitivity atmospheric pressure photoionization (APPI) has been also used (Schwudke et al., 2007). ECAPCI is known to be less susceptible to suppression of ionization than ESI, and detection limits in the low pg/mL range for most estrogens can be attained (Penning et al., 2010).

Stable isotope dilution LC–MS/MS-based methodology is now accepted as the “gold standard” to quantify the low amounts of eicosanoids that are present in serum and plasma. Analyses are normally conducted using deuterium-labeled eicosanoid analogs as internal standards (ISTDs), which confer much greater specificity than structural analog ISTDs. Quantification is performed by constructing calibration curves for each analyte. Standard solutions of different concentrations are made through serial dilution with commercially available standard eicosanoids and are spiked with the same amount of the deuterium-labeled ISTD as the samples to be determined. It has been shown that when no stable isotope ISTD is used, the amount of ion suppression at different elution times can vary from sample to sample (Bonfiglio et al., 1999; King et al., 2000).

Sample cleanup is one of the most critical steps in eicosanoid analysis because interference coming from matrix and other isomers present in the biological fluids



**Figure 17.3** ECAPCI/HRMS analysis of 15(S)-HETE-PFB.

is a major challenge. Most protocols for sample cleanup involve solid-phase extraction (SPE) or liquid–liquid extraction (LLE). The original Folch et al. (1951) and Bligh and Dyer extraction (Bligh and Dyer, 1959) methods work the best for LLE and are used nowadays with minor modifications by different research groups. The principle of the method is based on a mixture of chloroform and methanol with water that would create a monophasic solution. When more water is added, the lipids will partition into the lower chloroform-rich phase and a layer of proteins will separate the aqueous phase that is on the top. The chloroform is nowadays many times replaced with the less toxic dichloromethane (Duflot et al., 2016), and the methanol can be replaced with different alcohols depending on the specific applications. It is worth mentioning that simple acidification of the aqueous biological samples can give faster protocols when is used in a biphasic extraction with ethyl acetate, ether, or methyl tert butyl ether (Mesaros et al., 2010). For SPE cleanup, the same type of cartridges can be used as it was for the LC columns. Reverse phase SPE is the preferred choice when not using a LLE method.

The availability of the [<sup>13</sup>C]-labeled ISTDs for all of the eicosanoid metabolites makes it possible to identify background contamination coming from extraction steps. It is particularly important to have blank samples with just the ISTDs added from the beginning that are then carried through the entire workflow, including the hydrolysis step—for the determination of the total eicosanoids, both esterified and free. The enzymes used for the hydrolysis of the esters, as well as the glassware used, can increase the background levels, which could interfere with the analyte signals, especially for the less abundant eicosanoids. In addition, it is advisable to test each ISTD individually and check for level of each analyte, since we encountered cases when one ISTD was contaminated with a different unlabeled eicosanoid. Sometimes derivatization is employed to increase the sensitivity for the low picomolar amounts of the eicosanoids. The carboxylic acid moiety can be easily transformed into an ester as in the case of the reaction with PFB bromides in the presence of a mild base (Lee et al., 2005b). The same cautionary steps need to be taken for the derivatization step, as with the cleanup. We have had a batch of derivatization reagents that was contaminated with PGD<sub>2</sub>, so this must be examined as well.

The analysis of the total (free + esterified) eicosanoids after the enzymatic or chemical hydrolysis is less challenging than the free eicosanoids since the levels of the total eicosanoids are higher. Caution should be taken to check for interferences from the enzymes. Most of the times, the free eicosanoids will give a better reading of the enzymatic processes since the hydrolysis can cause racemization. One way to avoid the drawbacks

associated with the hydrolysis would be to analyze intact esterified lipids. In this way it is easier to get a complete picture of the different classes of lipids containing esterified eicosanoids.

LC using reversed-phase solvents with microbore columns or normal phase solvents with chiral columns is now capable of separating complex mixtures of eicosanoid regioisomers and enantiomers. LC separations represent one of the most time-consuming aspects of eicosanoid analysis. Reducing the LC run time can have a profound impact on the number of assays that can be performed in a day. However, it is important that isomeric eicosanoids are separated from each other (like PGE<sub>2</sub> and PGD<sub>2</sub>). An example to demonstrate the need of separating the two PGs is shown in the chronic model of induced gastric ulcers by acetic acid (Motilva et al., 2005). COX-2 was expressed at 7 days in the mouse and was also associated with PGE<sub>2</sub> increase. Administration of ibuprofen and rofecoxib decreased the PGE<sub>2</sub> levels. Without having the two PGs chromatographically separated, one could not measure accurately the decrease in PGE<sub>2</sub> since PGD<sub>2</sub> levels are a few folds higher than PGE<sub>2</sub>. The decrease in PGE<sub>2</sub>, even though statistically significant, would not have been significant if it would be measured together with the high levels of PGD<sub>2</sub>. Thus, adequate chromatographic resolution between all the eicosanoids metabolites is required, which leads to longer chromatographic runs. This must be balanced against the accuracy that arises from the clear separation of all the isomers.

## 17.3 Examples of LC-MS Analysis of Lipids

### 17.3.1 Omega-6-Derived Eicosanoids

A study done by Fernandez Peralbo et al. (2013) described an SPE–LC–MS/MS methodology to monitor omega-6-derived eicosanoids, which are mediators of CVD (Peters-Golden and Henderson, 2007). Currently, many pharmacological treatments are aimed at manipulating the pathways that generate HETEs and EETs, such as through the epoxidation of AA by CYP450 enzymes. EETs have been shown to reduce ischemia (a type of reperfusion injury), induce vasodilation in the coronary circulation, and reduce vascular inflammation (Node et al., 1999). The most common type of CVD, coronary artery disease (CAD), occurs when sclerotic plaque builds in the arteries and causes a feedback loop in which a buildup of circulating lipids, followed by immune cells, leading to inflammation and further plaque deposition (Fuster et al., 2005). Although current methods of predicting and diagnosing CAD do exist, there is much room for improvement so as to prevent episodes of

acute myocardial infarction (AMI). Given the involvement of inflammatory eicosanoid metabolites in CAD, drugs that can target these signaling pathways (e.g., COX and LOX inhibitors) have shown promise in regulating the atherothrombotic process (Gleim et al., 2012). In an attempt to better understand and monitor the eicosanoid metabolites involved in CVD, Peralbo et al. developed an automated online SPE–LC–MS/MS technique with minimal involvement of the analyst in sample preparation. They compared the omega-6 FA eicosanoid metabolites from controls with those from patients with CAD, as well as from patients diagnosed with stable angina, AMI, or non-ST-segment elevation myocardial infarction (NSTEMI).

The methodology presented by Peralbo et al. can be completed in 33 min and, with the automated steps, allows for quicker sample preparation. Sample preparation was very fast, as serum was diluted 1:1 with water and spiked with 15-HETE-*d*<sub>8</sub> containing 0.01% formic acid before online SPE using a C18 cartridge. Reversed-phase UPLC using a C18 column was used. The limit of detection (LOD) ranged from 15 pg/mL to 8.6 ng/mL, while the limit of quantification (LOQ) ranged from 52 pg/mL to 28.6 ng/mL.

In comparing the concentrations of target eicosanoids between healthy controls and coronary lesion patients, they were able to quantitatively analyze 12 metabolites, of which 5 were above the quantitation limit in a significant number of samples. Between the two groups, the most significantly different family of metabolites were the TXs, which are involved in CVDs through effects on vasoconstriction, expression of endothelial adhesion molecules, and effects on the endothelial cells themselves (e.g., migration, proliferation, etc.) (Ali et al., 1993; Cheng et al., 2002; Ding and Murray, 2005; Nie et al., 2000). Moreover, when comparing the different coronary lesion patients with one another (NSTEMI, stable angina, AMI), it was seen that 12-HETE was significantly higher in AMI patients relative to patients with stable angina. Additionally, previous studies have shown the formation of this LOX metabolite during ischemia (Morykwas et al., 1992). The partial AUC parameter (1.4%) supported the specificity (90%) and sensitivity (36%) of this analysis using a receiver operating characteristic (ROC) curve. These results, although focused on the differences between AMI and stable angina patients, can still help to diagnose and prevent more severe AMI in patients who have already developed stable angina.

### 17.3.2 Docosahexaenoic Acid (DHA)

A methodology developed by Derogis et al. (2013) for docosahexaenoic acid (DHA) [22:6 n-3] could be easily applied to eicosanoids. Their study is worth mentioning

since it comprises all the necessary steps for eicosanoid detection starting with preparation of the standards and their purification. DHA is an FA that is highly enriched in the mammalian brain and, due to its strong presence in synaptosomal membranes and synaptic vesicles, is thought to be a key player in maintaining normal central nervous system function (Salem et al., 2001). In particular, changes in DHA oxidation or overall quantity are associated with the development and progression of neurodegenerative diseases (Bazan et al., 1984; Horrocks and Yeo, 1999; Lukiw and Bazan, 2008). Because of the six double bonds present in DHA, this FA is highly susceptible to oxidation, either enzymatically through COX, LOX, and CYP450 or nonenzymatically through ROS and transition metal ion interactions (Bazan et al., 1984; Cheng and Li, 2007; Fer et al., 2008; Serhan et al., 2002; Yin et al., 2011). Both of these mechanisms can result in a large variety of oxidative metabolites, specifically through the production of DHA hydroxide (HDoHE) and hydroperoxide (HpDoHE) isomers (Aveldano and Sprecher, 1983; Bazan et al., 1984; Cheng and Li, 2007; Fer et al., 2008; Sapieha et al., 2011; Serhan and Petasis, 2011; Serhan et al., 2002, 2009; Sun et al., 2007; Westphal et al., 2011; Yin et al., 2011). Given the importance of discerning between various isomers and understanding their roles in the development of neurological diseases, Derogis et al. developed a sensitive analytical method using LC–MS/MS for the detection and quantification of 12 major HpDoHE and HDoHE isomers in biological systems.

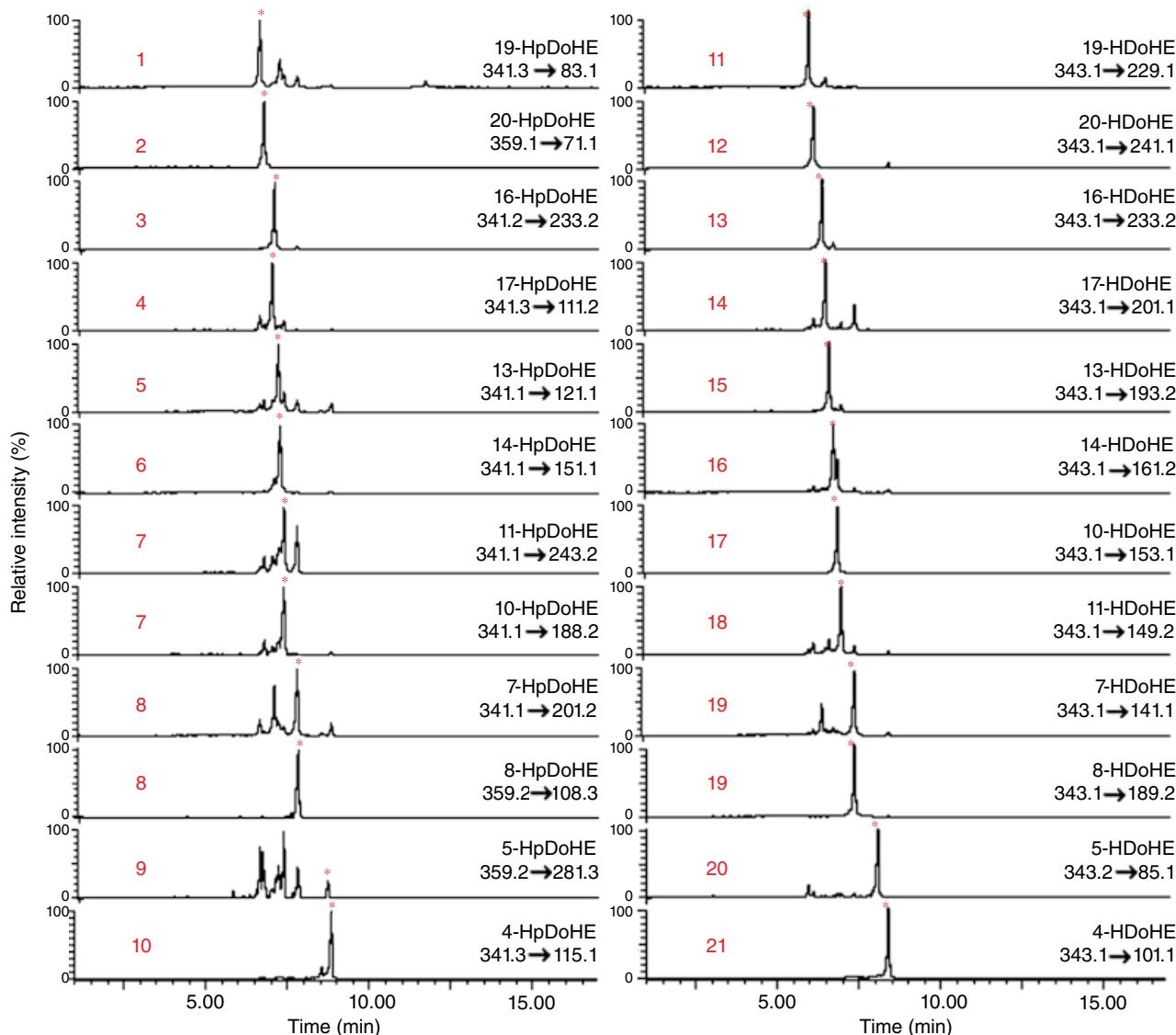
In order to prepare the HpDoHE and HDoHE standards, HpDoHE was synthesized by photooxidizing DHA in an O<sub>2</sub>-rich atmosphere with methylene blue as the photosensitizer (Miyamoto et al., 2003). Then, HpDoHE was converted to HDoHE through reduction with NaBH<sub>4</sub> (Terao et al., 1988), and the HpDoHE concentration is confirmed by iodometry (Buege and Aust, 1978).

An initial screening for the chromatographic method utilized the Waters Corp. Empower 3 chromatography data software and LC system. The parameters considered included the number of peaks, a resolution greater than 0.80, and a tailing smaller than 2.0. Moreover, four different reversed-phase LC columns (HSS T3, HSS PFP, BEH C8, and CSH Phenyl-Hexyl) were tested. Additionally, pH values at 2.5, 3.5, and 10 were evaluated, and methanol and acetonitrile were used as the organic modifiers. Three temperatures (25, 35, and 40°C) and organic solvent gradients (between 80 and 100%) from 5 to 10 min were examined. Ultimately, the most effective setup was found to be a BEH C8 column (with a length of 100 mm and particle size of 1.7 μm) eluted with a gradient solvent system of 0.1% ammonium hydroxide in water (pH 10) and 18% methanol in acetonitrile at 0.5 mL/min. Ammonium hydroxide is many times a

better choice for the negative mode since formic acid is causing suppression of ionization in negative mode.

A comparison of the experimentally observed fragments and the theoretically expected fragments enabled the identification and characterization of each HpDoHE and HDdoHE isomer (Figure 17.4). With the MS/MS spectra of each HpDoHE and HDdoHE isomer, and considering the intensity and specificity of the fragment ions observed, an MRM method was developed. In order to increase sensitivity of fragment ion detection and maintain an adequate number of data points across each chromatographic peak, the analysis was split into two separate methods (analyzing the HpDoHE and HDdoHE isomers separately).

The method was applied to the detection of the basal levels of the isomers in plasma and brain samples. Plasma and brain samples were obtained from rats, and the brain cortex samples were homogenized in phosphate-buffered saline (PBS) solution. To extract the lipids from all the samples, the Bligh and Dyer method was used with slight modifications (Bligh and Dyer, 1959; Derogis et al., 2013). As expected from prior recovery studies, the HpDoHE isomers were not detected in the tested biological samples. However, 11 of the HDdoHE isomers were detected in the rat plasma, and all 12 were detected in the brain samples. The most abundant isomer, the 14-HDdoHE isomer (product of 12-LOX), was present at 6–10 times higher than any of the other isomers. This



**Figure 17.4** Chromatograms of individual SRM transitions selected for each HpDoHE and HDdoHE isomer seen in a mixture from rat brain and plasma samples. Source: Reprinted with permission from Derogis et al. (2013).

was not the first time that this trend has been seen, as Gomolka et al. (2011) tested whole blood samples from mice and also found much higher levels of 14-HDoHE.

Despite being the first study to describe the detection of the HDoHE isomers in brain homogenates at basal conditions, Derogis et al. still managed to detect all 12 of these isomers using their specified LC–MS/MS method. Additionally, in what is likely a result of the higher concentrations of DHA in the nervous tissue than in the blood, they found higher levels of HDoHE in the brain samples. Within the brain samples, the 4-, 20-, 14-, and 11-HDoHE isomers were highest out of the 12 HDoHE isomers (and detected at similar levels to one another). The reason for high levels of 14- and 11-HDoHE may be due to the fact that the most predominant LOX in the brain is 12-LOX that mediates formation of these two metabolites, while larger accumulation of the 20- and 4-HDoHE isomers could be due to oxidation of DHA (Bendani et al., 1995; Hambrecht et al., 1987; Kim et al., 1990; Lyberg and Adlercreutz, 2006). This new LC–MS/MS technique will allow more efficient analysis of hydro( pero)xy derivatives of DHA and AA and thus further study of oxidative lipidomics in biological systems.

### 17.3.3 N-Acylethanolamines (NAEs) and Eicosanoids

*N*-acylethanolamines (NAEs) are a separate class of endogenous bioactive lipid mediators that are composed of a fatty acyl conjugated (through the amide bond) to ethanolamine (Buczynski and Parsons, 2010; Ueda et al., 2010). NAEs, together with eicosanoids, are important lipid-signaling molecules involved in many critical biological processes, such as immunological control of cell recruitment, inflammation, pain signaling, blood pressure response, and fever (Buczynski et al., 2009; Funk, 2001). Their metabolites have even been implicated in a wide range of complex diseases including neurodegeneration, cystic fibrosis, rheumatoid arthritis, atherosclerosis, and cancer (Calder and Zurier, 2001; Harmon et al., 2010; Hyde and Missailidis, 2009; Pierre et al., 2009; Rodriguez-Antona and Ingelman-Sundberg, 2006; Weitz et al., 2010). Hydrolysis of NAEs leads to the formation of the free FAs and ethanolamine and, more importantly, can result in the inactivation of downstream NAEs signaling. Pharmacological targeting of the small molecule metabolites in the NAEs pathways has garnered significant interest, although further knowledge of these mechanisms through enhanced identification and quantification of these relevant metabolites is needed (Ahn et al., 2009; Buczynski et al., 2010; Karbarz et al., 2009; Piomelli et al., 2006; Reinke, 1992; Wang et al., 2009).

In one study, Dumiao et al. (2011) improved a high-throughput LC–MS/MS technique, which allowed

monitoring and quantification of over 100 unique eicosanoid and 36 NAE species in a single analysis. The sample preparation was the same for both eicosanoid and NAEs analysis and consisted of suspending the murine sample in 10% methanol in water, spiking with deuterated ISTD solution, and extracting lipid metabolites using polymerized solid reversed-phase extraction columns. The decision to pursue the SPE technique stems from the fact that this method is more suitable for processing larger numbers of samples (Golovko and Murphy, 2008). The columns were then washed with 100% methanol followed by water, and then samples were loaded before washing with 10% methanol to remove nonspecific hydrophobic chemical species and salts.

Identical LC analysis was used for the eicosanoids and NAEs, with the exception of different buffer systems. The samples were lyophilized and then resuspended in (for eicosanoids) solvent A<sub>EICOS</sub>, which consists of water:acetonitrile:acetic acid (70:30:0.02), or solvent B<sub>EICOS</sub>, which consists of acetonitrile:isopropyl alcohol (50:50). For NAEs, they were resuspended in either a solvent consisted of water:acetonitrile:acetic acid (70:30:0.1) and 1 g/L ammonium acetate or a solvent consisted of acetonitrile:isopropyl alcohol:acetic acid (45:45:10) and 1 g/L ammonium acetate. Using these various methodologies, samples could be subjected to either both in series or each one alone. After analyzing a sample for eicosanoids, the addition of a water:acetonitrile:acetic acid (70:30:0.1) and 5 g/L ammonium acetate solution to the sample would make it suitable for analysis in positive ion mode. NAEs were run on a Luna reversed-phase C8 column, while eicosanoids were run on a Synergi reversed-phase C18 column.

An ABI/Sciex 4000 QTrap<sup>TM</sup> hybrid triple-quadrupole linear ion trap mass spectrometer equipped with a Turbo V ion source was used. By using MRM pairs at scheduled retention time (scheduled MRM), the total number of metabolites monitored without sacrificing data quality was increased. Because all eicosanoids are derived from polyunsaturated fatty acids (PUFAs), they contain a conserved terminal carboxyl moiety that is easily deprotonated, which allows their detection in negative ion mode. NAEs, on the other hand, ionize better in positive ion mode since they contain conserved amide nitrogen. Thus, eicosanoids were detected in negative electrospray ion mode, while NAEs were analyzed in positive electrospray ion mode. Upon creating the calibration curve through serial dilution and analysis, the LOD in the range between 0.1 and 1 pg for eicosanoids, and generally at 0.1 pg for NAEs, in a few instances, it ranged from 10 to 100 pg. These sensitivity disparities were due to efficiency of the parent to daughter ion transitions.

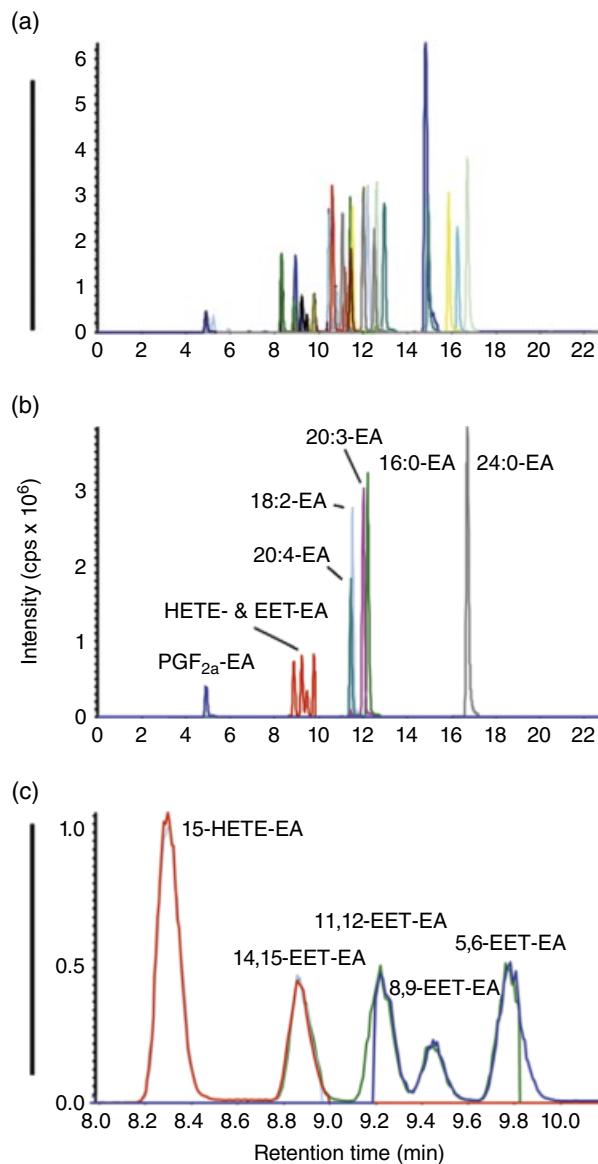
As is generally the case in LC–MS, there will be some amount of lipid metabolite that is lost due to the

extraction process or degradation, and thus an appropriate ISTD will allow the researcher to properly account for these variations. Effective identification and detection of a scheduled MRM pair with a corresponding retention time from a reversed-phase HPLC gradient elution for a particular lipid metabolite is also largely dependent on the available ISTDs. The methodology for eicosanoids proposed by Dumla et al. contained 171 MRM pairs (for the eicosanoids and related metabolites, monitored in a single 25 min LC-MS/MS run) of which 141 were metabolites and 30 were deuterated ISTDs (along with 36 metabolites and 4 ISTDs for NAEs). The chromatographic separation is critical when attempting to monitor a large number of structurally similar metabolites because many analytes share MRM transitions, as observed for 39 occurrences for eicosanoids and 19 cases for NAEs (Figure 17.5).

The importance of monitoring a large number of various lipid biological mediators is due to the fact that we do not yet know the full roles of individual mediators and thus cannot draw accurate conclusions using a small number of metabolites. As a demonstration of the application of their methodologies to tissue samples, Dumla et al. globally monitored the changes in eicosanoids and NAEs in rat cerebral spinal fluid (CSF) and lumbar spinal cord sections after injection with a hyperalgesia-inducing agent. The researchers found elevated levels of AA-derived COX and 12-LOX metabolites, corresponding to the increased stress levels in rats and thus oxidation states of these metabolites. There were also fewer metabolites detected overall in the CSF when compared to the spinal cord samples, in particular the NAEs samples, which was not found in the CSF at all. Moreover, there were temporal differences, such as PGE<sub>2</sub>, being elevated at different times in the CSF and spinal cord. Ultimately, the methodology proposed by Dumla et al. not only is adaptable to other studies of metabolites but also allows for more thorough large-scale analyses.

#### 17.3.4 Arachidonic Acid (AA)

An interesting study by Mangal et al. (2011) explored the AA pathways as a means for monitoring the use of steroids in racehorses. For instance, glucocorticoids (GCCs) are used as anti-inflammatory drugs in the racing industry but are sometimes abused so that the horse will be able to tolerate greater levels of pain, leading to potential injury to the horse and/or the rider (Mangal et al., 2011). After testing several different extraction conditions, using both LLE and SPE, Mangal et al. optimized their methodology and chose LLE as the sole procedure due to the similar recovery for all analytes, yet lower costs and faster speeds that are characteristic of LLE. The researchers developed a chloroform/isopropanol-based



**Figure 17.5** Targeted lipidomics using LC-ESI/MS/MS for analysis of eicosanoids derivatives. Injected sample is a primary standard solution of eicosanoids. (a) 102 eicosanoid sMRM pairs were extracted. (b) Several eicosanoid sMRM pairs were chosen and extracted. (c) Magnified image of HETE and HODE metabolite chromatographs. Source: Dumla et al. (2011). Reproduced with permission of Elsevier.

LLE methodology for preparation of blood samples (efficiency of 13–100%), along with a stable isotope dilution LC-MS/MS assay, for analyzing eicosanoid concentrations in horses (Deems et al., 2007).

To stimulate eicosanoid production, they exogenously added AA to the whole blood samples of healthy non-racing horses, or treated the whole blood with calcium ionophore (CI) A23187, in order to release endogenous

AA from esterified lipids, and then analyzed plasma eicosanoids. Additionally, they evaluated the effects of dexamethasone (DEX), a glucocorticoid type of anti-inflammatory drug, in the pretreatment of equine whole blood for inhibition of AA or CI A23187-mediated formation of eicosanoids. In an attempt to reduce generation of artifacts from eicosanoids, which is highly problematic during the extraction process due to transition metal ion-mediated Fenton chemistry, the researchers added deferoxamine to chelate residual transition metal ions in the whole blood sample. LC was conducted using a Kinetex C<sub>18</sub> column (length 100 mm, particle size 2.6 μ) coupled to a 4000 QTrap™ mass spectrometer in negative ion mode. After testing the addition of varying levels of formic acid as a mobile phase additive, it was discovered that 0.02% formic acid yielded the highest ionization sensitivity for eicosanoids. It was showed that 9-HETE, 12-HETE, and 15-HETE were increased when the whole blood was treated with CI A23187 or AA but decreased when pretreated with DEX. CI A23187 treatment greatly increased the concentrations of the eicosanoids LTE<sub>4</sub> and LTB<sub>4</sub>, which result from 5-LOX activity. While DEX pretreatment significantly decreased these metabolites, AA treatment had no effect on their production. TXB<sub>2</sub>, a downstream product of COX-1 enzyme activity, was increased by AA and CI A23187 treatment and decreased by DEX down to or even below baseline levels. However, the other downstream COX products PGE<sub>2</sub>, PGD<sub>2</sub>, and prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) were not

affected by CI A23187 treatment, but did increase with AA and decrease with DEX treatment.

Given that DEX can inhibit eicosanoid production, it has the capacity to reduce joint inflammation. The study done by Mangal et al. is currently being used to elucidate the mechanisms of eicosanoid production following glucocorticoid administration in racehorses and can be used to identify eicosanoid changes that lead to excess pathological risks associated with inflammation (Mangal et al., 2011).

## 17.4 Summary and Future Directions

Overall, the various methodologies presented here demonstrate recent advancement in the field of lipidomics and allow for more efficient quantitation and monitoring of lipid metabolites. There is increasing interest in the use of high-resolution mass spectrometers for lipid analysis. This will certainly contribute substantially to the improvement of the existing assays by improving specificity and sensitivity. By using HR–MS/MS, a recent report (Mazaleuskaya et al., 2016) improved the sensitivity of a previously published method by more than 50 times. With the enhanced capability to identify and monitor increased numbers of lipids and their metabolites, it will become possible to dissect the complicated cellular pathways and reveal valuable biomarkers for diagnosis and treatment of diseases.

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## 18

# Targeted LC–MS Quantification of Androgens and Estrogens for Biomarker Development

Daniel Tamae\*

*Department of Systems Pharmacology and Translational Therapeutics, Center of Excellence in Environmental Toxicology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA*

\* Current address: Department of Chemistry and Biochemistry, California State University, Northridge, CA, USA

## 18.1 Introduction

Androgen and estrogen signaling is required for sexual development, normal human physiology, and reproduction. Both classes of steroid hormones are derived from cholesterol. Biosynthesis occurs in the gonads, adrenal glands, and peripheral tissues such as the breast, the prostate, and the placenta. Androgens and estrogens share the basic cyclopentanophenanthrene 4-ring structure with its precursor, cholesterol (Figure 18.1). The carbon atoms are assigned with numbers and the rings with letters as dictated by standard convention. The oxidative demethylation of the C19 androgens by aromatase, mainly testosterone (T) and  $\Delta^4$ -androstene-3, 17-dione ( $\Delta^4$ -AD), leads to the formation of the C18 estrogens, estradiol ( $E_2$ ) and estrone ( $E_1$ ), respectively, with an aromatic A-ring.

Estrogen and androgen biosynthesis and signaling drive the progression of hormone-dependent tumors such as that of the breast and the prostate. These pathways can be impacted by exposure to endocrine-disrupting chemicals (EDCs). Dysregulated estrogen and androgen biosynthesis or signaling can lead to endocrine diseases such as polycystic ovary syndrome, hypogonadism, and androgen insensitivity syndromes. The careful study of steroid hormone biosynthesis and signaling requires sensitive and specific methods for the quantification of estrogens and androgens and their metabolites.

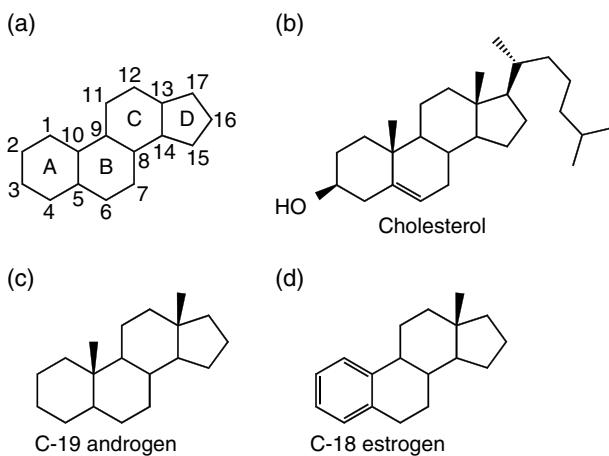
### 18.1.1 History of Estrogen and Androgen Quantification

In the late 1960s, Beiser and Erlanger pioneered the use of antibodies to estimate levels of T and  $E_1$ , among other steroid hormones (Beiser and Erlanger, 1967).

This was soon followed by the development of the radioimmunoassay (RIA) approach for the quantification of steroid hormones by Berson and Yalow (Yalow, 1973). The use of classical RIAs has given way to easier antibody-based platforms such enzyme-linked immunosorbent assays (ELISAs), which continue to be valuable tools in endocrine research. These methods can be conducted with relative ease and without specialized training and thus have been the method of choice for many labs engaged in endocrinology research. However, a number of studies have shown that ELISAs are inaccurate at the low concentration range of androgens or estrogens (i.e., androgen quantification under castrate conditions in prostate cancer or estrogens in postmenopausal females) (Wang et al., 2004; Huhtaniemi et al., 2012; Ohlsson et al., 2013). The antibodies are prone to matrix effects and there is the potential for cross-reactivity of any given antibody to multiple metabolites.

Thus for applications where sensitivity and specificity is paramount, RIAs and ELISAs have been replaced by liquid chromatography tandem mass spectrometry (LC–MS/MS) methods. This transition has been taking place in laboratories at academic centers as well as at commercial clinical laboratory operations in the United States (Stanczyk and Clarke, 2010). Stable isotope internal standards facilitate the development of stable isotope dilution methods that, when coupled with LC–MS/MS, are the gold standard for the quantification of small molecules such as androgens and estrogens. Great strides in steroid hormone quantification have been made using stable isotope dilution gas chromatography mass spectrometry (GC–MS) methods (Choi and Chung, 2015), but these studies are outside the scope of this book and will therefore not be discussed.

The Endocrine Society has authored position papers on the utility, limitations, and pitfalls of antibody-based



**Figure 18.1** (a) The general cyclopentanophenanthrene 4-ring structure of steroid hormones is presented with the carbon numbering convention and letter assignments for the rings. (b) The structure of cholesterol. (c) The general structure of the C19 androgens. (d) The general structure of the C18 estrogens.

and mass spectrometric assays for T and E<sub>2</sub> (Rosner et al., 2007; Handelsman and Wartofsky, 2013; Rosner et al., 2013), and the Centers for Disease Control and Prevention (CDC) and leaders in the field have called for and organized efforts for the harmonization and standardization of steroid hormone quantification (Stanczyk et al., 2007; Bhasin et al., 2008; Vesper et al., 2008). Rigorous studies have also been conducted with estrogens and androgens and their respective metabolites in order to ensure assay reproducibility (Fuhrman et al., 2014; Buttler et al., 2015). Here, we review the state of the art in LC-MS/MS quantification of estrogens and androgens and highlight case studies illustrating the utility of steroid hormones and their metabolites as biomarkers.

### 18.1.2 Androgen Biosynthesis and Metabolism

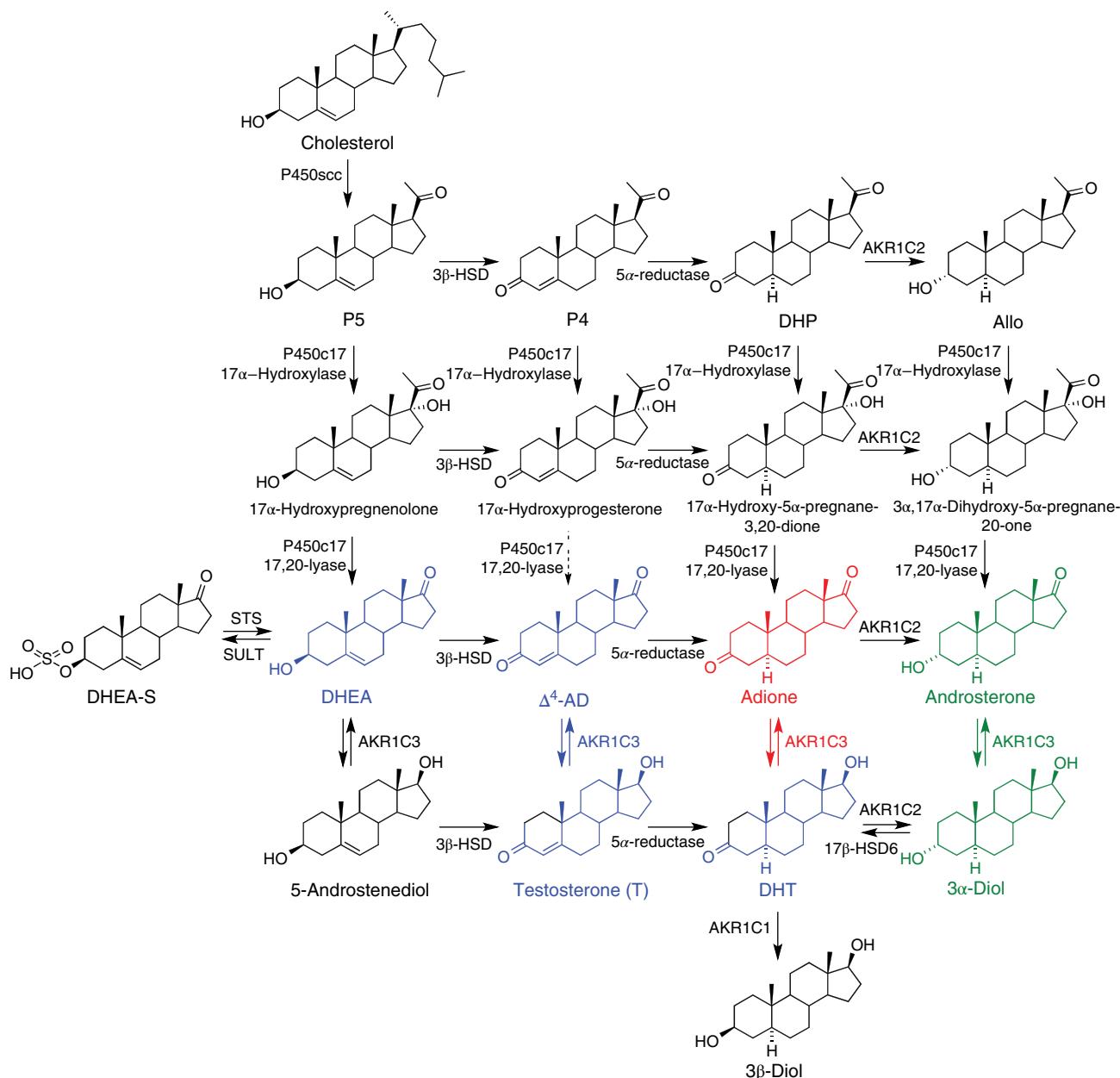
The adrenal glands and the testes are the predominant sites of androgen production. Androgen biosynthesis begins with the precursor, cholesterol, which is converted to the C-21 steroid, pregnenolone (P5), via the cytochrome P450 side-chain cleavage (P450scc) enzyme (Figure 18.2). The dual activity of cytochrome P450 17 (P450c17), the 17 $\alpha$ -hydroxylase and 17,20-lyase, acts upon P5 successively to convert it to the C-19 steroid, dehydroepiandrosterone (DHEA). DHEA is then converted by 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) to  $\Delta^4$ -AD, which is converted to T via the activity of the 17 $\beta$ -hydroxysteroid dehydrogenase type 5 (17 $\beta$ -HSD5), also known as aldo-keto reductase 1C3 (AKR1C3). T is then converted via 5 $\alpha$ -reductase to 5 $\alpha$ -dihydrotestosterone (DHT). This is the canonical pathway to DHT biosynthesis (DHEA  $\rightarrow$   $\Delta^4$ -AD  $\rightarrow$  T  $\rightarrow$  DHT). Alternatively,

$\Delta^4$ -AD is converted to 5 $\alpha$ -androstane-3,17-dione (Adione) via 5 $\alpha$ -reductase. Adione is then a substrate for AKR1C3 conversion to DHT. This has been referred to as the bypass pathway as it does not require T for the production of DHT (DHEA  $\rightarrow$   $\Delta^4$ -AD  $\rightarrow$  Adione  $\rightarrow$  DHT) (Sharifi, 2012). Finally, during male development, the backdoor pathway is known to play a key role (Fluck et al., 2011). The backdoor pathway is comprised of conversion of P5 to progesterone (P4) via 3 $\beta$ -HSD, which is then converted to 5 $\alpha$ -dihydroprogesterone (5 $\alpha$ -DHP) via 5 $\alpha$ -reductase activity. Then, 5 $\alpha$ -DHP is converted to allopregnanolone (Allo) via aldo-keto reductase 1C2 (AKR1C2) activity. Allo is then converted by P450c17 to androsterone (A), which is converted to 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (3 $\alpha$ -Diol) via AKR1C3 activity. The final step, the conversion of 3 $\alpha$ -diol to DHT, is catalyzed by 17 $\beta$ -hydroxysteroid dehydrogenase type 6 (17 $\beta$ -HSD6). The backdoor pathway is the third potential pathway to DHT biosynthesis (P5  $\rightarrow$  P4  $\rightarrow$  5 $\alpha$ -DHP  $\rightarrow$  Allo  $\rightarrow$  A  $\rightarrow$  3 $\alpha$ -Diol  $\rightarrow$  DHT). T and DHT elicit their biological effects primarily through the binding of the androgen receptor (AR), which leads to its translocation into the nucleus and the expression of androgen-regulated genes. We should note that the clinical convention for the reporting of units of concentration of androgens is in ng/dL. Therefore, an effort has been made in this chapter to use units of ng/dL for ease of comparison of different methods.

### 18.1.3 Estrogen Biosynthesis and Metabolism

The ovaries and the placenta are the predominant sites of estrogen production. Estrogens are also produced in other sites in the peripheral tissue such as in the breast tumor. In determining breast cancer risk in postmenopausal women, it is informative to understand the biosynthesis and metabolism of estrogens in the breast tissue. Estrogen can be manufactured from progesterone (P4). The 17 $\alpha$ -hydroxylase and 17,20-lyase activity of P450c17 converts P4 to the C-19 precursor steroid,  $\Delta^4$ -AD. Aromatase converts  $\Delta^4$ -AD to E<sub>1</sub>. Alternatively,  $\Delta^4$ -AD can be converted to T via AKR1C3, which can then be converted to E<sub>2</sub> via aromatase. E<sub>2</sub> can also be converted to E<sub>1</sub> via the action of 17 $\beta$ -hydroxysteroid dehydrogenase-2 and -14 (17 $\beta$ -HSD2, 14). Conversely, E<sub>1</sub> can be reduced to E<sub>2</sub> by AKR1C3 or 17 $\beta$ -hydroxysteroid dehydrogenase-1, -7, and -12 (17 $\beta$ -HSD1, 7, 12). E<sub>2</sub> is a ligand for the nuclear estrogen receptor- $\alpha$  and - $\beta$  (ER- $\alpha$ /- $\beta$ ) and binding results in dimerization and translocation of the estrogen receptor (ER) into the nucleus, where it binds to ER-specific binding sites and regulates gene expression.

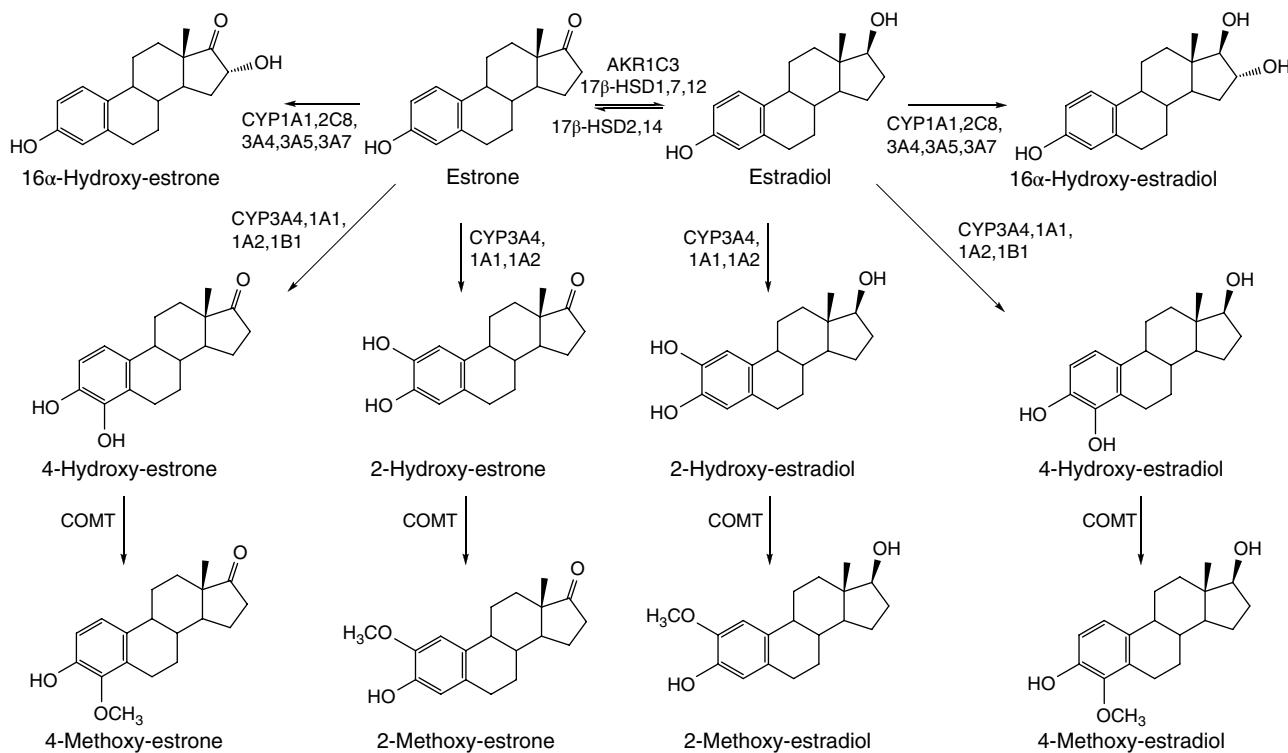
E<sub>1</sub> and E<sub>2</sub> are metabolized to their catechol estrogens via members of the cytochrome P450 (CYP) enzyme



**Figure 18.2** The androgen biosynthetic pathway. The active ligands for the androgen receptor, testosterone (T), and 5 $\alpha$ -dihydrotestosterone (DHT) are manufactured from cholesterol. This scheme details the many pathways to the formation of T and DHT. The canonical pathway to the formation of DHT: cholesterol is converted by the P450scc enzyme to form the C21 steroid, pregnenolone (P5), which is a substrate for the P450c17 17 $\alpha$ -hydroxylase and 17,20-lyase activity to yield the C19 androgen, dehydroepiandrosterone (DHEA). DHEA is a substrate for 3 $\beta$ -HSD, which converts it to  $\Delta^4$ -AD, which is converted by AKR1C3 to form T. Finally, T is a substrate for 5 $\alpha$ -reductase to form DHT. The bypass pathway proceeds via 5 $\alpha$ -androstane-3,17-dione (Adione) and forms DHT through the action of AKR1C3, in a T-independent manner. There is also the backdoor pathway, whereby P5 is converted to progesterone (P4) via 3 $\beta$ -HSD, which is then converted to 5 $\alpha$ -DHP via the action of 5 $\alpha$ -reductase. Then, 5 $\alpha$ -DHP is converted to allopregnanolone (Allo) via AKR1C2 activity. Allo is then converted by P450c17 to androsterone (A), which is converted to 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (3 $\alpha$ -Diol) via AKR1C3 activity. The final step is the conversion of 3 $\beta$ -Diol to DHT, which is catalyzed by 17 $\beta$ -HSD6.

family (Figure 18.3). These catechol estrogens are substrates for catechol-O-methyltransferases (COMTs), which generate the 2- or 4-methoxy-estrogens. The measurement of the 2- and 4-methoxy-estrogens has

been used as a surrogate for their respective catechol estrogens, which may be informative in breast cancer risk assessment (Santen et al., 2015). There is also evidence to suggest that the 2-methoxy-estrogens are



**Figure 18.3** The estrogen metabolism pathway. The action of CYP1A1, 2C8, 3A4, 3A5, and 3A7 can convert the estrogens, estrone ( $E_1$ ), and estradiol ( $E_2$ ) to their  $16\alpha$ -OH metabolites. The activity of CYP3A4, 1A1, and 1A2 can convert the estrogens to the 2-OH metabolites, which are then substrates for the action of COMT to yield the respective 2-MeO metabolites. Similarly, the activity of CYP3A4, 1A1, 1A2, and 1B1 can convert the estrogens to the 4-OH metabolites, which are then substrates for the action of COMT to yield the respective 4-MeO-metabolites.

antiproliferative and protective against carcinogenesis (Eliassen et al., 2008; Arslan et al., 2009).

Estrogens can be metabolized via sulfotransferases (SULTs) and uridine diphosphate glucuronosyltransferases (UGTs) to form estrogen sulfates and glucuronides, respectively. Estrogen sulfates are the most abundant circulating estrogen conjugates, followed by the estrogen glucuronides. Estrone sulfate ( $E_1$ -S) is present in the circulation at roughly 10 times the level of unconjugated  $E_1$  (Pasqualini et al., 1989).  $E_1$  and  $E_2$  have been shown to be substrates for many members of the UGT1A family (Raftogianis et al., 2000; Guillemette et al., 2004). We should note that the clinical convention for the reporting of units of concentration of androgens is pg/mL. Therefore, an effort has been made to use units of pg/mL for ease of comparison of different methods.

## 18.2 Current Considerations in Biomarker Validation

A biomarker is an entity that can be quantified and whose level tracks with initiation, progression, and pharmacologic treatment of a disease state. Biomarkers may include, but are not limited to, gene transcripts,

metabolites, polysaccharides, nucleic acids, single nucleotide polymorphisms, and splice variants. There are also different types of biomarkers. Predictive biomarkers define a subpopulation of patients that are likely to respond to a particular therapy. Prognostic biomarkers define the likely outcome of the disease, irrespective of treatment. Pharmacodynamic biomarkers define an interaction between a drug and a target and may characterize both therapeutic and adverse effects. Biomarkers may also serve as surrogates for clinical end points such as patient survival (Drucker and Krapfenbauer, 2013).

The development of a bona fide clinical biomarker first requires bioanalytical method validation. The US Food and Drug Administration (FDA) has set forth guidelines on the validation of bioanalytical methods for biomarker candidates. These guidelines dictate metrics of specificity, accuracy, and precision that must be achieved in order to afford sufficient reliability and reproducibility (FDA, 2013). Similarly, bioanalytical assays for biomarkers should be confirmed for their performance with a “fit-for-purpose” approach, which means that assay validation should be tailored to meet the intended purpose of the biomarker study, with a level of rigor commensurate with the intended use of the data (Lee et al., 2006). Once the bioanalytical method has been validated, the

next phase of biomarker development is clinical application and validation. Close collaboration with clinicians and biostatisticians is required in order to design clinical trials that are statistically powered to validate the biomarker in the relevant patient cohort and to provide sufficient biofluid or tissue biopsy material. A balance must be struck between the clinical feasibility and cost of sampling the biofluid or tissue biopsy with the level of information that is expected to be gleaned from the study. Consideration must also be given to the circadian rhythm of the biomarker of interest and the potential effect of time of day collection of biospecimen on study outcomes (Brambilla et al., 2009; Stolze et al., 2015). Finally, LC-MS/MS methods are extremely sensitive, so extra care must be taken by all parties during the chain of collection, storage, processing, and analysis such that the samples are not contaminated.

### 18.3 Current Considerations in LC-MS Method Development

Stable isotope dilution coupled to LC-MS/MS is the state of the art in the quantification of small molecules and proteins. The exquisite specificity and sensitivity of the LC-MS/MS technology contribute to the superiority of this method over antibody-based platforms. Specificity is derived from chromatographic resolution, mass selective detection, and use of the mass transition of parent ion to fragment ion for the detection of each analyte. Sensitivity may vary based on the analyte and the instrumentation being used. The use of derivatization agents that result in increased ionization efficiency has been well established to increase the sensitivity of detection of the steroid derivative. An investment of time may be required during the method development for targeted quantification of estrogen and androgens. When done properly, a considerable return on investment can be derived by way of the robustness and ruggedness of the method. There are many considerations to take into account during the method development phase, and we will highlight the most relevant issues here.

#### 18.3.1 Chromatography

One of the challenges in quantifying endogenous small molecules such as androgens and estrogens is the presence of intermediates and metabolites that possess very similar chemical structures and have the same exact mass, for example, stereoisomers such as T and epitestosterone (epi-T). In resolving stereoisomers, the selectivity conferred by mass selective detection and the mass transition of the parent ion to fragment ion is rendered ineffective. Therefore, specificity of the method

will ultimately rest upon the chromatographic resolution of the analytes. We have achieved chromatographic resolution of the GirT hydrazones of T, epi-T, and Adione using the Kinetex XB-C18 column (Phenomenex, Torrance, CA), which employs a C18 stationary phase with isobutyl side chains and trimethylsilyl (TMS) end-capping. We have since applied the method to serum samples from patients undergoing androgen deprivation therapy (ADT) for prostate cancer in the total androgen pathway suppression (TAPS) trial and the neo-adjuvant abiraterone acetate (AA) trial. The chromatographic resolution of our method gave us and our clinical colleagues a high degree of confidence that measurement of all of the androgen metabolites reflected the true value (Tamae et al., 2013; Mostaghel et al., 2014; Taplin et al., 2014).

The introduction of ultra-performance liquid chromatography (UPLC) has greatly improved sensitivity, speed, and chromatographic resolution relative to methods that had been previously designed using high-performance liquid chromatography (HPLC). In a heads-up comparison of HPLC versus UPLC for the separation of phytoestrogens and tamoxifen, the UPLC method decreased the HPLC run time from 10 min down to 3.5 min, and the sharper UPLC peaks led to improved signal-to-noise ratios (S/N) that varied from 1 (no change) for dihydrodaidzein to a 9.8-fold increase in S/N for tamoxifen. All chromatography was done on Waters' ACQUITY UPLC system (Waters, Milford, MA). The UPLC separation was done on a 1 mm × 50 mm ACQUITY reversed-phase column with 1.7 μm particle size (Waters), and HPLC separation was done on a 2 mm × 150 mm Luna C18-2 column with 3 μm particle size (Phenomenex, Torrance, CA). Mass spectrometry was done via electrospray on Waters' Quattro Premier triple-quadrupole mass spectrometer (Waters) in positive ion mode. It is particularly interesting that the improvement in S/N varied depending on the analyte and was not simply arithmetic based on the theoretical changes in peak shape in moving from HPLC to UPLC (Churchwell et al., 2005).

#### 18.3.2 Direct Detection Methods

Direct detection by mass spectrometry is most amenable to estrogen and androgen conjugates such as glucuronides and sulfates. In normal physiology, steroids are conjugated by phase II enzymes, and this conjugation is a mechanism for the regulation of the biological activity of androgens and estrogens via their inactivation and clearance. The expression and activity of UGTs such as UGT2B7, UGT2B15, and UGT2B17 are the known players in the glucuronidation of androgens (Chouinard et al., 2007). These enzymes catalyze the transfer of the glucuronyl moiety from UDP-glucuronic acid to the androgen target, making the androgen hydrophilic and

thus readily excreted. A variety of direct detection methods have been established for the quantification of steroid glucuronides and sulfates.

Androsterone glucuronide (A-G), 5 $\alpha$ -androstane-3 $\alpha$ , 17 $\beta$ -diol-3-glucuronide (3 $\alpha$ -diol-3-G) and 5 $\alpha$ -androstane-3 $\alpha$ , 17 $\beta$ -diol-17-glucuronide (3 $\alpha$ -diol-17-G), and dehydroepiandrosterone sulfate (DHEA-S) were directly detected and quantified using deuterated stable isotope internal standards by electrospray ionization (ESI) tandem mass spectrometry using a Sciex API 3000 triple quadrupole equipped with TurbulonSpray (Labrie et al., 2006).

E<sub>1</sub>-S, estradiol sulfate (E<sub>2</sub>-S), DHEA-S, 16-OH-DHEA-S, 5-androstene-3 $\beta$ , 17 $\beta$ -disulfate (3 $\beta$ -Diol-S), and pregnenolone sulfate (P5-S) were directly detected and quantified using deuterated stable isotope internal standards. E<sub>1</sub>-S; E<sub>2</sub>-S; DHEA-S, 16-OH-DHEA-S, and A-S ; and P5-S were detected with sensitivity down to 0.4 ng/mL, 0.08 ng/mL, 1 ng/mL, and 0.5 ng/mL, respectively. ESI and atmospheric pressure chemical ionization (APCI) tandem mass spectrometry was done on the Thermo TSQ Quantum Ultra in negative ion mode to quantify sulfated steroids (Galuska et al., 2013).

Urinary testosterone glucuronide (T-G), epitestosterone glucuronide (epiT-G), A-G, etiocholanolone glucuronide (ET-G), norandrosterone glucuronide (NA-G), and noretiocholanolone glucuronide (NET-G) were quantified by direct detection using deuterated stable isotope internal standards. T-G and epiT-G were detected with sensitivity down to 125 ng/dL; A-G and ET-G, 2500 ng/dL; NA-G, 25 ng/dL; and NET-G, 12.5 ng/dL. ESI tandem mass spectrometry was conducted on a Thermo TSQ Quantum Discovery Max triple-quadrupole. Concordance of the LC-MS/MS results with GC/MS results was also examined (Pozo et al., 2008).

The direct detection of unconjugated androgens and estrogens is not optimal due to the lack of ionizable moieties on the steroid ring. However, there are some studies that have shown that direct detection of the unconjugated steroid is feasible. Atmospheric pressure photoionization (APPI) tandem mass spectrometry was conducted on an API-5000 triple quadrupole (Applied Biosystems/MDS SCIEX, Ontario, Canada) with deuterated stable isotope internal standards for the quantification of intact T with sensitivity down to 1 ng/dL and DHT to 5 ng/dL, along with E<sub>1</sub> to 1.5 pg/mL and E<sub>2</sub> to 2.5 pg/mL (Harwood and Handelsman, 2009).

Direct detection of androgens was also accomplished using ESI tandem mass spectrometry on a QTRAP 5500 (Applied Biosystems/MDS SCIEX) with deuterated internal standards for the quantification of T down to 0.625 ng/dL; DHT, 2.5 ng/dL;  $\Delta^4$ -AD, 1.25 ng/dL; DHEA, 25 ng/dL; and A, 50 ng/dL. Although good sensitivity was achieved for the majority of the androgens, the

sensitivity obtained for A showed the limitations of the direct detection approach with regards to sensitivity (Wilton et al., 2014).

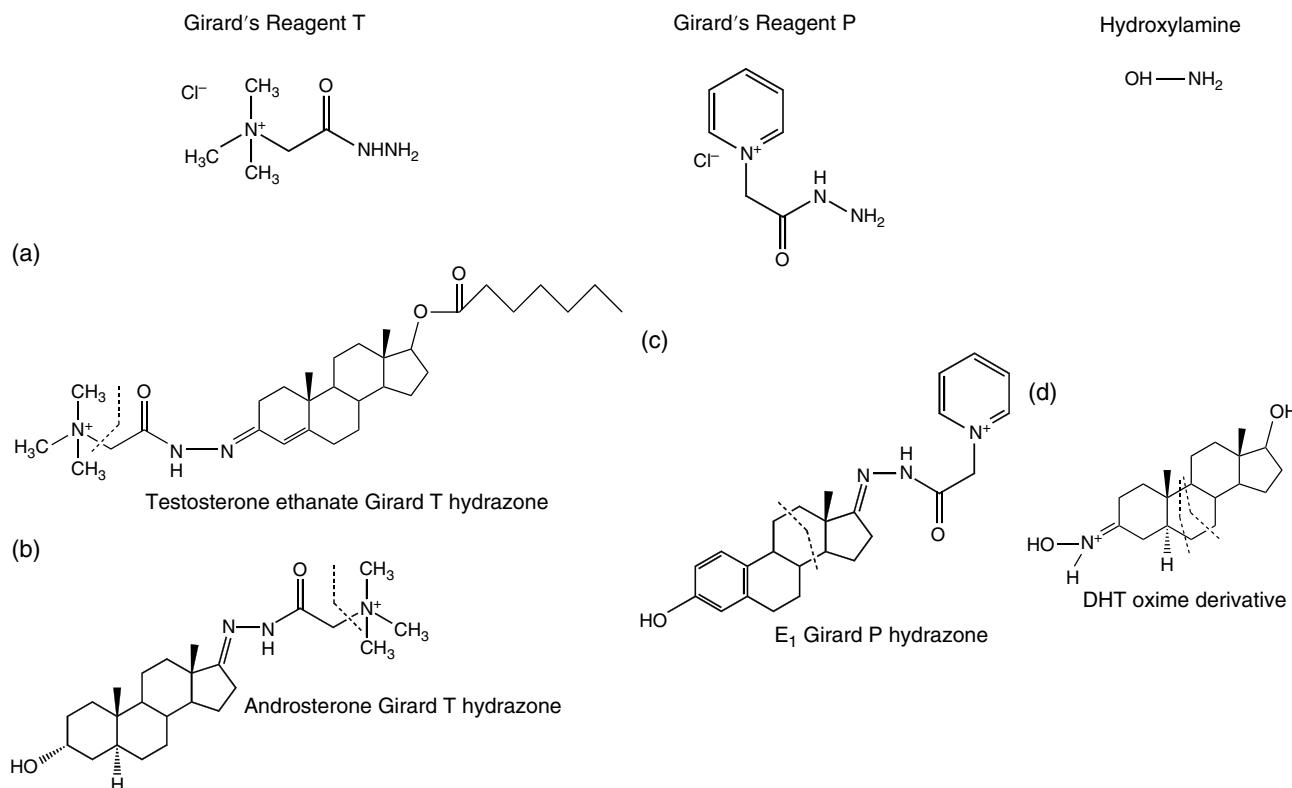
### 18.3.3 Derivatization Strategies

The sensitivity of the LC-MS/MS method is directly related to the ionization efficiency of the analyte of interest. Unconjugated steroids do not possess acidic or basic moieties and are therefore not optimized for methods that utilize soft atmospheric pressure ionization sources such as ESI, APCI, and APPI. Chemical derivatization is used to introduce a pre-ionized or easily ionizable moiety onto the analyte of interest and increase sensitivity, and there are many excellent reviews on this topic (Higashi and Shimada, 2004; Santa et al., 2007; Santa, 2011). Furthermore, the chemical derivative will often provide a mass transition to generate an intense product ion by collision-induced dissociation (CID), allowing for sensitive detection by LC-MS/MS. For the LC-MS/MS quantification of ketosteroids, (carboxymethyl)trimethylammonium chloride hydrazide or Girard's reagent T (GirT) (Shackleton et al., 1997; Johnson, 2005; Tamae et al., 2013), 1-(carboxymethyl)pyridinium chloride hydrazide or Girard's reagent P (GirP) (Griffiths et al., 2003; Rangiah et al., 2011), hydroxylamine (Kalhorn et al., 2007), and methoxyamine are among the derivatization agents frequently used (Figure 18.4). For the quantification of hydroxysteroids, fusaric acid (Yamashita et al., 2009b), 5-dimethylamino-1-naphthalenesulfonyl chloride or dansyl chloride (Dns-Cl) (Nelson et al., 2004), and picolinic acid (Yamashita et al., 2009a) have been used.

Pre-ionized moieties of the quaternary ammonium or pyridinium salts greatly increase ionization efficiency via the use of GirT and GirP reagents, respectively, and have been used to derivatize ketosteroids (Griffiths et al., 2003). Pioneering work on the application of the GirT derivative for the analysis of T esters was done in plasma for sport doping analysis (Shackleton et al., 1997). Our lab has since applied GirT and enzymatic hydrolysis to the quantification of the conjugated and unconjugated keto-androgens using <sup>13</sup>C<sub>3</sub>-labeled stable isotope internal standards. This approach has been used to quantify serum  $\Delta^4$ -AD, DHEA, T, epi-T, DHT, and A and their respective glucuronides and sulfates. T was detected with a sensitivity of 0.5 ng/dL; DHT, epi-T,  $\Delta^4$ -AD, and A, 2.5 ng/dL; and DHEA, 10 ng/dL. Keto-androgen GirT hydrazones were quantified by ESI on a Thermo TSQ Quantum triple quadrupole, and this method was applied to serum samples from prostate cancer patients enrolled in the TAPS trial (Tamae et al., 2013).

Pre-ionized GirP derivatives have been used to quantify E<sub>1</sub> and its metabolites in the serum of postmenopausal women (Figure 18.4). Derivatives were quantified on an

### Derivatization of keto-steroids



**Figure 18.4** Derivatization agents commonly used for ketosteroid detection and quantification via LC-MS/MS. Girard's reagent T (GirT) also known as (carboxymethyl)trimethylammonium chloride hydrazide, Girard's reagent P (GirP) also known as 1-(carboxymethyl) pyridinium chloride hydrazide, and hydroxylamine are a few examples of the commonly utilized derivatization agents for keto-steroids. (a and b) Androgen esters, androgens, and their metabolites have been quantified via pre-ionized Girard's reagent T hydrazones. (c) Estrogens and their metabolites have been quantified via pre-ionized Girard's reagent P hydrazones. (d) T and DHT have been quantified via pre-ionized hydroxylamine oximes. Dotted lines indicate collision-induced dissociation fragmentation point.

Eksigent ultra-2D nanoflow LC system (Eksigent Technologies, Dublin, CA, USA) in line with a Thermo Vantage TSQ triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a CaptiveSpray ion source (Michrom Bioresources Inc., Auburn, CA, USA). This approach obtained sensitivity down to 0.156 pg/mL for E<sub>1</sub>, 16 $\alpha$ -hydroxyestrone, 2-methoxyestrone, and 4-methoxyestrone using <sup>13</sup>C<sub>6</sub>-E<sub>1</sub>, <sup>13</sup>C<sub>6</sub>-2-methoxyestrone, and <sup>13</sup>C<sub>6</sub>-4-methoxyestrone internal standards. This study showed that there was no significant difference in E<sub>1</sub> and its metabolite levels in serum from postmenopausal smokers versus non-smokers. Further, when compared with 11 previous E<sub>1</sub> quantification trials, the mean serum E<sub>1</sub> in control subjects was two- to threefold lower than 7 of 11 of the studies (Rangiah et al., 2011).

ESI tandem mass spectrometry with deuterated stable isotope internal standards has been used to quantify 2-hydrazino-1-methylpyridine (HMP) pre-ionized derivatives of T and DHT down to a sensitivity of 1 ng/g of

tissue in 10 mg biopsy samples for both T and DHT (Higashi et al., 2005). LC-ESI-MS/MS with deuterated stable isotope internal standards was also used to quantify pre-ionized hydroxylamine derivatives of T and DHT and achieved sensitivity of 0.1nM of DHT (Kalhorn et al., 2007).

#### 18.3.4 Stable Isotope Standards

Stable isotope-labeled androgens and estrogens are required for quantification via LC-MS/MS. The standards are typically labeled with deuterium (<sup>2</sup>H) in lieu of hydrogen (<sup>1</sup>H) or carbon-13 (<sup>13</sup>C) in lieu of carbon-12 (<sup>12</sup>C). It is optimal to have three or more atoms exchanged for labeling purposes. High ionization temperatures have been found to lead to hydrogen-deuterium exchange of internal standards (Stokvis et al., 2005). Therefore, the preference should always be for stable isotope standards that contain <sup>13</sup>C atoms in the steroid of interest. However, not all steroids are commercially available as <sup>13</sup>C

standards. Cambridge Isotope Laboratories (Andover, MA, USA) has  $^{13}\text{C}_6$ -estrogen standards commercially available and Sigma-Aldrich (St. Louis, MO, USA) has  $^{13}\text{C}_3\text{-T}$  and  $^{13}\text{C}_3\text{-DHT}$ . In our laboratory, where we lacked commercially available standards, we have utilized recombinant enzymes to stereoselectively synthesize  $^{13}\text{C}_3$ -labeled androstanediols from  $^{13}\text{C}_3\text{-DHT}$  (Zang et al., 2016).

### 18.3.5 Hydrolysis of Conjugated Steroids

The quantification of steroid conjugates such as the glucuronides and sulfates has been discussed in the context of direct detection methods (Section 18.3.2). However, many in the field have also achieved conjugate quantification by using a de-conjugation approach via enzymatic hydrolysis using recombinant *E. coli*  $\beta$ -glucuronidase and arylsulfatase from a variety of sources (Wang et al., 2015). Once de-conjugated, the steroids can be analyzed by derivatization and subsequent LC-MS analysis. Several considerations should be taken into account when using this approach. First, there can be variability in the activity of the enzymes; therefore it is best to use a standard assay to assess the actual enzyme activity. We have found the p-nitrocatechol sulfate assay for arylsulfatase titration and the phenolphthalein-glucuronide assay for  $\beta$ -glucuronidase to be adequate for this purpose. Second, arylsulfatase sources may be contaminated with other steroid metabolizing enzymes. We, and others, have found *Helix pomatia* arylsulfatase from Sigma-Aldrich (St. Louis, MO, USA) to be contaminated with  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ -HSD) activity (Vanluchene et al., 1982; Messeri et al., 1984; Hauser et al., 2008). Abalone entrails arylsulfatase from Sigma-Aldrich was found to be free of  $3\beta$ -HSD. As with the analysis of unconjugated steroids, the addition of stable isotope internal standards at the earliest step of de-conjugation is necessary (Tamae et al., 2013).

## 18.4 Clinical Application of LC-MS Quantification of Estrogens and Androgens

The development and validation of an LC-MS method for the quantification of estrogens and androgens and their conjugates and metabolites is the first step in biomarker development. The second step is the clinical validation, which requires patient biofluids or tissue to ascertain specificity and sensitivity of the biomarker of interest. Here, we highlight some case studies on the clinical validation of targeted estrogen and androgen quantification as biomarkers.

### 18.4.1 Reference Ranges of Estrogens and Androgens

The establishment of the reference range of estrogens and androgens in healthy age-matched females and males is of vital importance for the diagnosis, treatment, and prevention of a wide range of endocrine disorders and diseases. At academic centers and commercial clinical laboratories, LC-MS/MS methods have begun to supplant antibody-based methods for the quantification of steroid hormones. As the measurement of serum T is prone to variability in samples from women and young children, reference ranges must be established using validated LC-MS/MS methods. The CDC has taken a lead role in the standardization and harmonization of LC-MS/MS quantification of T (Vesper et al., 2008; Vesper and Botelho, 2010). T has been quantified without derivatization using  $^{13}\text{C}_3\text{-T}$  internal standards and attained sensitivity down to 0.95 ng/dL and linearity to 1300 ng/dL (Wang et al., 2014). This method was applied to the 2011–2012 National Health and Nutrition Examination Survey (NHANES). This study analyzed 6746 serum samples from the NHANES cohort. The results established 10th–90th percentiles of T in adult men (20 years and older) as 150–698 ng/dL, in adult women (20 years and older) as 7.1–49.8 ng/dL, and in children (6–10 years old) as 1.0–9.5 ng/dL (Vesper et al., 2015). Separately, serum T levels were measured in Generation 3 of the Framingham Heart Study (FHS), which used 456 serum samples to establish 5th and 95th percentiles of T in adult men (19–40 years of age) as 406–1124 ng/dL. This study employed quality controls from the CDC harmonization program and used a hydroxylamine derivatization strategy with deuterated T internal standard and yielded sensitivity down to 2 ng/dL (Bhasin et al., 2011).

Estrogens and estrogen metabolites were measured in serum form a subcohort ( $n=895$ ) of the Breast and Bone Follow-up to the Fracture Intervention Trial (B~FIT,  $n=15,595$ ). Participants were postmenopausal and not on hormone therapy. The study established 10th and 90th percentiles of  $E_1$  in the subcohort as 102.3–768.8 pmol/L,  $E_2$  as 18.1–91.4 pmol/L, 2-hydroxyestrone (2-OH- $E_1$ ) as 38.2–140.8 pmol/L, 2-hydroxyestradiol (2-OH- $E_2$ ) as 7.5–25.5 pmol/L, 2-methoxyestrone (2-MeO- $E_1$ ) as 13.8–59.5 pmol/L, 2-methoxyestradiol (2-MeO- $E_2$ ) as 6.7–24.0 pmol/L, 4-hydroxyestrone (4-OH- $E_1$ ) as 6.4–21.5 pmol/L, 4-methoxyestrone (4-MeO- $E_1$ ) as 2.1–8.0 pmol/L, 4-methoxyestradiol (4-MeO- $E_2$ ) as 1.2–5.7 pmol/L, and 16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OH- $E_1$ ) as 17.8–69.2 pmol/L; other metabolites were also quantified (Dallal et al., 2016). The LC-MS/MS quantification of the estrogens and estrogen metabolites for this study utilized liquid/liquid extraction and a dansyl chloride derivatization approach; deuterated internal standards

were used to obtain sensitivity down to 8 pg/mL (26.5–29.6 fmol/mL). Calibration curves were linear over a 1000-fold concentration range (Xu et al., 2007).

Estrogen and estrogen metabolites were measured in serum from lean ( $n=12$ ) and obese ( $n=23$ ) prepubertal females. Interquartile ranges were reported as 1.8–8.7 pmol/L of E<sub>2</sub> in lean females and 1.8–17.1 pmol/L of E<sub>2</sub> in obese females, 117.2–468.9 pmol/L of total E<sub>1</sub> (conjugated + unconjugated E<sub>1</sub>) in lean females and 88.0–286.3 pmol/L of total E<sub>1</sub> in obese females, 1.7–4.3 pmol/L of 2-MeO-E<sub>2</sub> in lean females and 1.7–1.7 pmol/L of 2-MeO-E<sub>2</sub> in obese females, and 1.7–3.2 pmol/L of 16 $\alpha$ -OH-E<sub>1</sub> in lean females and 1.7–33.7 pmol/L in obese females; other metabolites were also measured but without significant difference between the two cohorts (Mauras et al., 2015). The LC-MS/MS method attained sensitivity down to 1 fg on column for E<sub>2</sub>, E<sub>1</sub>, 16 $\alpha$ -OH-E<sub>2</sub>, 16 $\alpha$ -OH-E<sub>1</sub>, 4-MeO-E<sub>2</sub>, 4-MeO-E<sub>1</sub>, 2-MeO-E<sub>2</sub>, and 2-MeO-E<sub>1</sub> and down to 10 fg on column for 4-OH-E<sub>2</sub>, 4-OH-E<sub>1</sub>, 2-OH-E<sub>2</sub>, and 2-OH-E<sub>1</sub>. The use of pre-ionized Girard P and N-methyl-pyridinium sulfate derivatives and <sup>13</sup>C-labeled internal standards and microflow liquid chromatography contributed to the exquisite sensitivity of the method (Wang et al., 2015).

#### **18.4.2 Estrogens in Postmenopausal Women and Low Androgens in Aging Men**

The process of aging leads to the natural decline of serum estrogens in females and serum androgens in males. This decline in circulating steroid hormone levels has been associated with health conditions such as osteoporosis and cardiovascular disease in postmenopausal females and diminished libido in older males, as well as diabetes and obesity. In order to quantify estrogens in postmenopausal females and aging males, N-methyl-pyridinium-3-sulfonyl (NMPS) has been used as a derivatizing agent, and the derivatives of E<sub>2</sub> and its metabolites were quantified using stable isotope dilution LC-ESI-MS/MS with exquisite sensitivity down to 0.5 pg/mL (Wang et al., 2015). In aging males, hormone replacement therapy for low T has gained traction, but questions remain as to the potential risks associated with treatment. In order to interrogate the effects of hormone replacement therapy, it is imperative to monitor the serum T levels in order to correlate these levels with clinical end points. Treatment monitoring of T supplementation has been conducted with quantification done by LC-ESI-MS/MS method with sensitivity down to 1 ng/dL for T (Swerdloff et al., 2015).

#### **18.4.3 Estrogens and Breast Cancer**

Breast cancer is the most frequently diagnosed cancer in females in the developing world. It is a hormone-driven

disease. Therapies have been developed to target estrogen biosynthesis via inhibition of aromatase and the antagonism of the ER. First-line therapy in ER-positive breast cancer includes tamoxifen, which acts as an ER antagonist via metabolism to active metabolites such as 4-hydroxy-tamoxifen via cytochrome P450 2D6 and 3A4 (CYP2D6, 3A4); secondary metabolism can also lead to N-desmethyl-4-hydroxy-tamoxifen (Wu et al., 2009). These metabolites have one to two orders of magnitude greater affinity for the ER compared with tamoxifen (Lim et al., 2005). First-line therapy for postmenopausal ER-positive breast cancer also includes the aromatase inhibitors (AIs) such as exemestane, anastrozole, and letrozole. These agents competitively inhibit aromatase, which shuts down the extra-gonadal conversion of androgens to estrogens. These drugs are not approved for premenopausal breast cancer patients, as the ovaries are the primary source of estrogens in these patients. Second-line therapy for ER-positive breast cancer includes fulvestrant, which is a selective estrogen receptor degrader (SERD) and is approved for patients that have progressed after other endocrine therapies (Howell et al., 2002). The use of LC-MS/MS methods may be useful in assessing therapeutic response to these therapies. One study assessed the efficacy of exemestane treatments by quantifying serum E<sub>1</sub>, E<sub>2</sub>, and E<sub>3</sub> using dansyl derivatization and underivatized conjugates, achieving a sensitivity of 1 pg/mL for the E<sub>2</sub>, 2 pg/mL for both the E<sub>1</sub> and E<sub>3</sub>, 6 pg/mL for E<sub>1</sub>-3S, 8 pg/mL for E<sub>2</sub>-3S, 15 pg/mL for E<sub>1</sub>-3G, 21 pg/mL for E<sub>3</sub>-3S, 22 pg/mL for E<sub>2</sub>-3G, 25 pg/mL for E<sub>3</sub>-16G, 33 pg/mL for E<sub>2</sub>-17G, 58 pg/mL for E<sub>3</sub>-3G, and 950 pg/mL for E<sub>2</sub>-3,17G (Zhao et al., 2014).

Estrogen metabolites may also contribute to carcinogenesis via DNA adduct formation (Cavalieri et al., 1997; Bolton et al., 1998). In this setting, the quantification of serum E<sub>2</sub>, E<sub>1</sub>, and their respective 2- and 4-methoxy-estrogens when coupled with longitudinal clinical trials may greatly improve breast, endometrial, and ovarian cancer risk assessment (Santen, 2008; Dallal et al., 2016; Trabert et al., 2016).

The use of the stable isotope dilution approach, in conjunction LC-ESI-MS/MS, has facilitated the specific and sensitive quantification of estrogens and their metabolites. The estrogens themselves are not readily ionizable by soft ionization techniques such as ESI or APCI. This challenge has been surmounted by chemical derivatization.

The pre-ionized N-methyl pyridinium-3-sulfonyl (NMPS) estrogen derivatives have facilitated ultrasensitive quantification with sensitivity for E<sub>2</sub>, 16 $\alpha$ -OH-E<sub>2</sub>, 4-MeO-E<sub>2</sub>, and 2-MeO-E<sub>2</sub> down to 0.5 pg/mL and with sensitivity for 4-OH-E<sub>2</sub> and 2-OH-E<sub>2</sub> down to 5 pg/mL (Wang et al., 2015). The pre-ionized N-methyl-nicotinoyl

(NMN) estrogen derivatives have been quantified using an ESI method with exquisite sensitivity down to 0.4 pg/mL for E<sub>2</sub> and 0.4 pg/mL for E<sub>1</sub> (Yang et al., 2008). The picolinoyl (P) estrogen derivatives have been quantified using an ESI method with sensitivity down to 0.5 pg/mL for E<sub>2</sub> and 1.0 pg/mL for E<sub>1</sub> (Yamashita et al., 2007). The pentafluorobenzyl (PFB) estrogen derivatives have been quantified using an electron capture atmospheric pressure chemical ionization mass spectrometry (ECAPCI-MS) method with sensitivity for E<sub>1</sub> and E<sub>2</sub> down to 5 pg/mL (Penning et al., 2010). GirT estrogen derivatives have been quantified using an ESI method with a sensitivity of 0.6 fg (2.2 amol) on column (Blair, 2010). The dansyl derivatization of 15 estrogen metabolites has achieved sensitivity down to 40 pg/mL (Xu et al., 2005; Falk et al., 2013). A variety of methods have been developed, validated, and applied to quantify a number of estrogens and their metabolites using different LC-MS/MS approaches (Table 18.1).

#### 18.4.4 Androgens and Prostate Cancer

Prostate cancer is a hormone-driven disease. As such, intermediate- and high-risk disease is frequently treated with ADT. First-line therapy includes leuproreotide, which is a luteinizing hormone releasing hormone agonist (LHRH) (Garnick, 1984), and bicalutamide, which is an AR antagonist (Furr et al., 1987). Second-line therapy includes AA, which is a cytochrome P450 17 $\alpha$ -hydroxylase and 17,20-lyase (P450c17) inhibitor (de Bono et al., 2011), and enzalutamide (Enza), which is an AR antagonist (Scher et al., 2012). The ADT armamentarium continues to grow in an effort to combat castration resistance and drug resistance and in some cases, minimize off-target effects such as with apalutamide (ARN-509), an AR antagonist that minimizes side effects such as seizures that have been reported in some patients being treated with Enza (Rathkopf et al., 2013). Galerone (TOK-001) is a 17,20-lyase inhibitor that also inhibits AR and leads to its degradation, but does not lead to mineralocorticoid and glucocorticoid excess, therefore does not require co-treatment with prednisone as AA does (Yu et al., 2014). ODM-201 is a nonsteroidal AR antagonist and has been found to inhibit several AR mutants that arise in patients such as F876L, in addition to wild type AR (Fizazi et al., 2014).

In the clinic, the efficacy of these agents is monitored using radiographic imaging of the tumor and quantification of the serum biomarker, prostate-specific antigen (PSA). Serum and tissue androgens may serve as biomarkers for monitoring the efficacy of the growing repertoire of ADT agents. A number of different LC-MS/MS methods for the quantification of androgens have been developed, validated, and applied to clinical specimens (Table 18.2). Serum is a convenient matrix because

it can be drawn serially before, during, and after treatment and informs the clinician as to the gonadal and adrenal output of potent androgens and their precursors and metabolites. Tumor tissue is informative because there may be selective uptake and intratumoral metabolism of androgen precursors or de novo biosynthesis of androgens within the tumor, which may result in completely different levels from that found in the serum (Titus et al., 2005; Stanbrough et al., 2006; Cai et al., 2011; Labrie, 2015). However, the use of tumor tissue is limited to the time of diagnostic biopsy, and the tissue recovered at the time of radical prostatectomy will be a mix of stromal tissue and non-tumor, adjacent tissue. The amount of tissue is also limited as it must be analyzed by clinical pathology, necessitating an exquisitely sensitive method in order to quantify castrate levels of androgens in milligram quantities of tissue material.

Many patients undergoing ADT inevitably develop castration-resistant prostate cancer (CRPC), whereby the tumor recurs or metastasizes and serum PSA levels resurge despite castrate levels of serum T and DHT. In order to better understand mechanisms of castration resistance, our lab has pioneered a novel, stable isotope dilution method for the quantification of the C19 keto-androgens using GirT as a derivatization agent and developed a strategy to process the unconjugated as well as the conjugated androgens (glucuronides and sulfates). This method was validated and applied to the quantitation of serum androgens from patients enrolled in three clinical trials of intense ADT in high-risk or metastatic castrate-resistant prostate cancer (mCRPC): the TAPS trial, the neo-adjuvant AA trial, and the AA + dutasteride trial (Mostaghel et al., 2014; Taplin et al., 2014). The data showed the clear benefit of having an agent that inhibits P450c17, whether it was non-specifically via ketoconazole or specifically with AA. These agents, when coupled with first-line therapies, resulted in a much more intense androgen deprivation as determined by quantification of adrenal androgen precursors. The data also led to an observation that although serum DHEA-S is greatly reduced by 90–95% after treatment with AA, a significant amount remains. Our hypothesis is that this DHEA-S depot may conspire with elevated androgen biosynthetic enzymes to drive a subset of mCRPC tumors (Tamae et al., 2015).

The quantification of C19 hydroxy-androgens has been worked out in our lab using a picolinic acid derivatization method (Zang et al., 2016). The derivatization as androgen P esters has allowed for the quantification of 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (3 $\alpha$ -diol) and 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (3 $\beta$ -diol) and their respective conjugates. Application of both the GirT for derivatization of the keto-androgens and picolinic acid for the derivatization of the hydroxy-androgens will allow for

**Table 18.1** LC-MS methods for targeted estrogen quantification.

Analyte	Ionization	LOQ pg/mL	Number of steroids	Internal standard	Reference
$E_2$	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
	ESI	0.5	2	$^{13}C$	Yamashita et al. (2007)
	ESI	1	12	Deuterated	Zhao et al. (2014)
	APPI	2.5	4	Deuterated	Harwood and Handelsman (2009)
	ECAPCI	5	8	Deuterated	Penning et al. (2010)
	ESI	8	10	Deuterated	Xu et al. (2007)
	ESI	40	15	Deuterated	Xu et al. (2005)
$E_1$	ESI	0.156	4	$^{13}C$	Rangiah et al. (2011)
	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
	ESI	1	2	$^{13}C$	Yamashita et al. (2007)
	APPI	1.5	4	Deuterated	Harwood and Handelsman (2009)
	ESI	2	12	Deuterated	Zhao et al. (2014)
	ECAPCI	5	8	Deuterated	Penning et al. (2010)
	ESI	8	10	Deuterated	Xu et al. (2007)
$E_3$	ESI	40	15	Deuterated	Xu et al. (2005)
	ESI	2	12	Deuterated	Zhao et al. (2014)
2-OH- $E_2$	ESI	5	12	$^{13}C$	Wang et al. (2015)
	ESI	8	10	Deuterated	Xu et al. (2007)
	ESI	40	15	Deuterated	Xu et al. (2005)
2-OH- $E_1$	ESI	5	12	$^{13}C$	Wang et al. (2015)
	ESI	8	10	Deuterated	Xu et al. (2007)
	ESI	40	15	Deuterated	Xu et al. (2005)
2-MeO- $E_2$	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
	ESI	8	10	Deuterated	Xu et al. (2007)
	ESI	40	15	Deuterated	Xu et al. (2005)
2-MeO- $E_1$	ESI	0.156	4	$^{13}C$	Rangiah et al. (2011)
	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
	ESI	8	10	Deuterated	Xu et al. (2007)
	ESI	40	15	Deuterated	Xu et al. (2005)
4-OH- $E_2$	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
	ESI	40	15	Deuterated	Xu et al. (2005)
	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
4-OH- $E_1$	ESI	8	10	Deuterated	Xu et al. (2007)
	ESI	40	15	Deuterated	Xu et al. (2005)
	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
4-MeO- $E_2$	ESI	8	10	Deuterated	Xu et al. (2007)
	ESI	40	15	Deuterated	Xu et al. (2005)
	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
4-MeO- $E_1$	ESI	0.156	4	$^{13}C$	Rangiah et al. (2011)
	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
	ESI	8	10	Deuterated	Xu et al. (2007)
	ESI	40	15	Deuterated	Xu et al. (2005)
16 $\alpha$ -OH- $E_2$	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
16 $\alpha$ -OH- $E_1$	ESI	0.156	4	$^{13}C$	Rangiah et al. (2011)
	ESI	0.5	12	$^{13}C$	Wang et al. (2015)
	ESI	8	10	Deuterated	Xu et al. (2007)
	ESI	40	15	Deuterated	Xu et al. (2005)

**Table 18.2** LC-MS methods for targeted androgen quantification.

Analyte	Ionization	LOQ (ng/dL)	Number of steroids	Internal standard	Reference
T	ESI	0.5	10	<sup>13</sup> C	Tamae et al. (2013)
	ESI	0.625	5	Deuterated	Wilton et al. (2014)
	ESI	0.95	1	<sup>13</sup> C	Wang et al. (2014)
	APPI	1	4	Deuterated	Harwood and Handelsman (2009)
	APCI	1	1	Deuterated	Swerdloff et al. (2015)
	APCI	2	1	Deuterated	Bhasin et al. (2011)
	ESI	2.5	9	<sup>13</sup> C	Zang et al. (2016)
DHT	ESI	2.5	10	<sup>13</sup> C	Tamae et al. (2013)
	ESI	2.5	5	Deuterated	Wilton et al. (2014)
	APPI	5	4	Deuterated	Harwood et al. (2009)
	ESI	6.25	9	<sup>13</sup> C	Zang et al. (2016)
DHEA	ESI	6.25	9	<sup>13</sup> C	Zang et al. (2016)
	ESI	10	10	<sup>13</sup> C	Tamae et al. (2013)
	ESI	25	5	Deuterated	Wilton et al. (2014)
$\Delta^4$ -AD	ESI	1.25	5	Deuterated	Wilton et al. (2014)
	ESI	2.5	10	<sup>13</sup> C	Tamae et al. (2013)
A	ESI	2.5	10	<sup>13</sup> C	Tamae et al. (2013)
	ESI	6.25	9	<sup>13</sup> C	Zang et al. (2016)
	ESI	50	5	Deuterated	Wilton et al. (2014)
epi-T	ESI	2.5	10	<sup>13</sup> C	Tamae et al. (2013)
	ESI	6.25	9	<sup>13</sup> C	Zang et al. (2016)
epi-A	ESI	6.25	9	<sup>13</sup> C	Zang et al. (2016)
3 $\alpha$ -diol	ESI	2.5	9	<sup>13</sup> C	Zang et al. (2016)
3 $\beta$ -diol	ESI	6.25	9	<sup>13</sup> C	Zang et al. (2016)
5-Adiol	ESI	2.5	9	<sup>13</sup> C	Zang et al. (2016)
T-G	ESI	1	10	<sup>13</sup> C	Tamae et al. (2013)
	ESI	125	6	Deuterated	Pozo et al. (2008)
DHT-G	ESI	2.5	10	<sup>13</sup> C	Tamae et al. (2013)
DHEA-G	ESI	10	10	<sup>13</sup> C	Tamae et al. (2013)
A-G	ESI	2.5	10	<sup>13</sup> C	Tamae et al. (2013)
	ESI	2500	6	Deuterated	Pozo et al. (2008)
epiT-G	ESI	125	6	Deuterated	Pozo et al. (2008)
ET-G	ESI	2500	6	Deuterated	Pozo et al. (2008)
NA-G	ESI	25	6	Deuterated	Pozo et al. (2008)
NET-G	ESI	25	6	Deuterated	Pozo et al. (2008)
DHEA-S	ESI	10	10	<sup>13</sup> C	Tamae et al. (2013)
	ESI/APCI	100	6	Deuterated	Galuska et al. (2013)

the quantification of the entire C19 androgen metabolome. It will also serve to facilitate our investigations into the mechanisms of castration resistance and drug resistance, which continues to claim the vast majority of prostate cancer related deaths. Specifically, the

quantification of 3 $\alpha$ -diol and its conjugates will allow us to interrogate the backdoor pathway, and the quantification of 3 $\beta$ -diol and its conjugates will allow for the understanding of the inactivation of DHT via the action of AKR1C1 (Figure 18.2).

## 18.5 Conclusion and Perspective

The targeted quantification of androgens and estrogens by LC-MS/MS has advanced greatly in the last two decades. The application of a variety of easily ionized and pre-ionized derivatization agents has greatly increased the sensitivity of LC-MS/MS methods. The commercial availability of more  $^{13}\text{C}$ -labeled androgens and estrogens has overcome the technical issues associated with deuterated internal standards. The introduction of microflow LC systems and the transition from HPLC to UPLC methods have improved peak shapes, further increasing sensitivity of methods, while also decreasing run times. The introduction of new mass spectrometer platforms has also increased the exquisite selectivity and sensitivity of existing methods. Newer generation mass spectrometers such as the Thermo Q-exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific) has

been used with GirP derivatization of androgens, and we have found an increase in sensitivity by an order of magnitude or more compared with previously established methods (Wang et al., 2015). The cumulative increase in sensitivity will facilitate the quantification of hormones in tissue samples from precious specimens such as patient-derived xenograft (PDX) mouse models of prostate and breast cancer, biopsy material from human patients prior to therapy and at time of radical prostatectomy or mastectomy, and other biofluids such as bone marrow aspirates to investigate sites of metastases. This capability will improve our understanding of systemic and intratumoral androgen and estrogen levels and allow for the interrogation of their roles in driving drug resistance and metastasis. Perhaps with this improved understanding of the role of steroid hormones in breast cancer risk and prostate cancer progression, we can begin to move these assays into the clinic and establish their biomarker potential.

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## 19

### Steroid Biomarkers

*Mike (Qingtao) Huang<sup>1</sup>, Shefali Patel<sup>2</sup>, and Zhongping (John) Lin<sup>3</sup>*

<sup>1</sup> Clinical Pharmacology, Akros Pharma Inc., Princeton, NJ, USA

<sup>2</sup> Bioanalytical & Pharmacokinetics, Janssen Research & Development, LLC, Spring House, PA, USA

<sup>3</sup> Bioanalytical and Biologics Services, Frontage Laboratories, Exton, PA, USA

#### 19.1 Introduction

Steroids are a group of lipids with complex structures containing four interlocking rings of a hydrogenated cyclopentanophenanthrene ring system; three of the rings contain six carbon atoms each and the fourth ring contains five. Steroids play pivotal roles in body biochemistry, and they include steroid hormones such as the gonadal or sex steroids, corticosteroids, glucocorticoids, and mineralocorticoids; vitamins of the D group; and sterols such as cholesterol.

This chapter will mainly focus on the current discussions of some of the sterols and glucocorticoids as biomarkers and their corresponding bioanalysis by LC–MS/MS. For sterols, our discussions will mainly focus on 4 $\beta$ -hydroxycholesterol (4 $\beta$ -OHC) and 24S- and 27-hydroxycholesterols (24S-OHC and 27-OHC). For glucocorticoids, our discussions will mainly focus on cortisol and 6 $\beta$ -hydroxycortisol (6 $\beta$ -HC). The structures of these compounds and their main functions in body are shown in Table 19.1.

#### 19.2 Sterols as Endogenous Biomarkers and Their Quantitation

Oxysterols are 27-carbon oxidized cholesterol-related molecules with additional hydroxyl, carbonyl, epoxy, hydroperoxy, or carboxyl moieties. Because of their ability to pass cell membranes and the blood–brain barrier at a faster rate than cholesterol itself, they are also important as transport forms of cholesterol. In addition, oxysterols have been ascribed a number of important roles in connection with cholesterol turnover, atherosclerosis, apoptosis, necrosis, inflammation, immunosuppression, and development of gallstones and been reported as

biomarkers (Leoni, 2009; Poli et al., 2013). The major oxysterols present in human circulation or tissues arise via enzymatic cholesterol oxidation processes (Russell, 2000; Schroepfer, 2000; Björkhem and Diczfalussy, 2002). Another important source of oxysterols is nonenzymatic autoxidation of cholesterol mediated mainly by lipid peroxides or free oxygen radicals (Brown et al., 2000; Lyons and Brown, 2001). This section will mainly discuss three of the oxysterols (4 $\beta$ -OHC, 24S-OHC, and 27-OHC) as biomarkers and their quantitation by LC–MS/MS. Table 19.2 briefly summarizes the main published assays for the quantitation of 4 $\beta$ -OHC, 24S-OHC, and 27-OHC using LC–MS/MS.

##### 19.2.1 4 $\beta$ -OHC as a P450 3A4/5 Endogenous Biomarker

It is important to evaluate drug–drug interactions (DDIs) at the early stage of drug development to avoid potential costly termination of drug candidates at the late stage of drug development due to DDI-mediated safety or efficacy concerns (Zhou et al., 2005; Sinz et al., 2008). Cytochrome P450 3A enzymes (CYP3A) have been used as common potential targets for DDI when multiple drugs are co-administrated to patients due to the fact that CYP3A are responsible for the metabolism of more than 50% of marketed drugs (Yu et al., 2014; Chu et al., 2009). The evaluation of induction or inhibition of CYP3A activity has been routinely used to predict clinically relevant DDI. Normally for a DDI study, a probe that is a CYP450 substrate (or a mixture of substrates) is administrated to evaluate CYP450 enzyme activity (Turpault et al., 2009). The disadvantages of such studies include complex (and also costly) study design and challenges in using probe drugs for some specific patient populations (such as pediatric and geriatric subjects, transplant recipients, and cancer patients) where the

**Table 19.1** Chemical structures and major functions of  $4\beta$ -hydroxycholesterol ( $4\beta$ -OHC), 24S- and 27-hydroxycholesterols, cortisol, and  $6\beta$ -hydroxycortisol.

Compound name	Chemical structure	Main functions in the body
Cortisol		<ul style="list-style-type: none"> <li>• Cortisol increases blood sugar through gluconeogenesis</li> <li>• Suppress the immune system</li> <li>• Aid in the metabolism of fat, protein, and carbohydrates</li> <li>• Decreases bone formation</li> <li>• Biological marker of stress, anxiety, and depression</li> </ul>
$6\beta$ -hydroxycortisol		<ul style="list-style-type: none"> <li>• Cortisol is metabolized to <math>6\beta</math>-hydroxycortisol by the human cytochrome CYP 3A4</li> <li>• P450 3A is predominantly responsible for cortisol <math>6\beta</math>-hydroxylase activity in human liver microsomes</li> <li>• The metabolic ratio (MR) of the concentration of <math>6\beta</math>-hydroxycortisol (<math>6\beta</math>-OHC) to cortisol (MR <math>6\beta</math>-OHC/cortisol) in human urine had been proposed as an endogenous marker for CYP3A activity</li> <li>• Acts as an endogenous marker for CYP3A4/5 activity</li> </ul>
$4\beta$ -hydroxycholesterol		
24S-hydroxycholesterol		<ul style="list-style-type: none"> <li>• Plays a central role in CNS sterol homeostasis</li> <li>• The most abundant hydroxycholesterol in the brain</li> <li>• Serum or plasma levels may reflect brain developmental and neuropathological changes associated with Alzheimer's disease (AD), Huntington's disease, and multiple sclerosis</li> </ul>
27-Hydroxycholesterol		<ul style="list-style-type: none"> <li>• Plays a role in sterol efflux from cells</li> <li>• Acts as a selective estrogen receptor modulator (SERM) function as a ligand for nuclear receptors, liver X receptors (LXR), and farnesoid X-activated receptors</li> <li>• Serum or plasma concentrations ratio of the brain-specific cholesterol metabolite 24S-OHC to the peripheral metabolite 27-OHC may be useful surrogate markers for neurodegenerative diseases including Alzheimer's disease, Huntington's disease, HIV-associated neurocognitive disorders, and multiple sclerosis</li> </ul>

administration of probe drugs may be detrimental to patient safety. Therefore, using endogenous biomarker instead of probe substrate to evaluate DDI not only will simplify the study design but also can have potential benefits from both financial point of view and patient's safety aspect.

$4\beta$ -OHC has been proposed as a new endogenous biomarker for cytochrome P450 3A4/5 (CYP3A4/5) activity with potential use in drug development. Bodin et al. initially reported (Bodin et al., 2001, 2002) that  $4\beta$ -OHC, a metabolite of cholesterol, was formed through the

enzyme activity of CYP3A4/5. Their data indicated that patients treated with the antiepileptic drugs phenobarbital, carbamazepine, or phenytoin, which are known to induce CYP3A, had highly elevated levels of  $4\beta$ -OHC plasma concentration. Their study (Bodin et al., 2002) on the metabolism of  $4\beta$ -OHC in healthy volunteers has also shown that the elimination of  $4\beta$ -OHC from the circulation was much slower than for other oysterols, possibly due to slow  $7\alpha$ -hydroxylation. Kanebratt et al. (2008) found out that rifampicin/rifampin, an anti-tuberculosis drug and a well-known inducer of CYP3A

**Table 19.2** Published LC–MS methods for the quantitation of 4 $\beta$ -OHC, 24S-OHC, and 27-OHC.

Analyte	Platform and author	Sample matrix	Sample volume ( $\mu$ L)	Curve range	Derivatization
4 $\beta$ -OHC	LC–APPI–MS/MS van de Merbel et al. (2011)	Human plasma	400	10–250 nM	No derivatization
4 $\beta$ -OHC	LC–ESI–MS/MS Goodenough et al. (2011)	Human plasma	50	2–500 ng/mL	Dipicolinyl ester
4 $\beta$ -OHC	LC–ESI–MS/MS Xu et al. (2013)	Human and mouse plasma	5	5–500 ng/mL	Dipicolinyl ester
4 $\beta$ -OHC	LC–ESI–MS/MS Huang et al. (2014)	Human plasma	50	5–500 ng/mL	Dipicolinyl ester
24S-OHC and 27-OHC	LC–APCI–MS Burkard et al. (2004)	Human plasma	100	40–400 ng/mL	No derivatization
24S-OHC and 27-OHC	LC–ESI–MS/MS Venkata et al. (2014)	Human serum	500	10–1000 ng/mL	No derivatization
24S-OHC	LC–APCI–MS/MS Sugimoto et al. (2015)	Human plasma	200	1–100 ng/mL	No derivatization

activity, increased 4 $\beta$ -OHC in a dose-dependent manner in a study with 24 health subjects. In that study, rifampicin was administered at doses of 20, 100, and 500 mg daily for 2 weeks, resulting in 1.5-, 2.5-, and 4-fold induction of 4 $\beta$ -OHC, respectively. Josephson et al. (2008) studied some antiretroviral drugs on the impact of 4 $\beta$ -OHC levels in plasma. Their results indicated that the median plasma 4 $\beta$ -OHC level increased by 46 ng/mL in patients treated with efavirenz. In contrast, the median plasma 4 $\beta$ -OHC level for patients given ritonavir-boosted atazanavir decreased 9.4 ng/mL and those given ritonavir-boosted lopinavir decreased by 5.8 ng/mL. Efavirenz is an inducer of CYP3A, whereas the ritonavir-boosted regimens are inhibitors of CYP3A. Wide et al. (2008) investigated the time course of the increase in plasma 4 $\beta$ -OHC concentration during treatment of pediatric patients with the CYP3A-inducer carbamazepine. The results indicated that the increase in 4 $\beta$ -OHC concentration continued for several weeks after the completion of induction of CYP3A4/5. Diczfalussy et al. (2009) investigated the elimination of 4 $\beta$ -OHC from the circulation following CYP3A induction with rifampicin. The results indicated that for the highest dose, 500 mg/day, while 4 $\beta$ -OHC concentrations increased significantly during the treatment, the concentrations of 4 $\alpha$ -hydroxycholesterol (4 $\alpha$ -OHC) were not influenced by rifampicin treatment, indicating that 4 $\alpha$ -OHC was not a product of CYP3A4/5-catalyzed metabolism. Their results also indicated that an unexpectedly long half-life of elimination (about 17 days) was found for 4 $\beta$ -OHC. This long half-life results in small variations within subjects in plasma concentration and is

an advantage when 4 $\beta$ -OHC is used as a marker for CYP3A activity during steady-state conditions. In contrast, the long half-life excludes 4 $\beta$ -OHC as a marker for rapid changes in CYP3A activity. Yang and Rodrigues (2010) reported the pharmacokinetic models of how the long half-life of 4 $\beta$ -OHC influenced the time course of CYP3A induction and inhibition. Using the half-life of 17 days, simulation curves for carbamazepine and rifampicin induction were very similar to the curves of the original publications (Wide et al., 2008; Diczfalussy et al., 2009). Furthermore, the simulations showed that it was possible to resolve weak, moderate, potent, and no inducers within 2 weeks of dosing. On the other hand, simulations indicated that at least 2 weeks of dosing would be needed to detect the potent inhibition of CYP3A (maximal ~40% decrease in 4 $\beta$ -OHC plasma levels). Greater differentiation of weak, moderate, and potent CYP3A inhibitors would require a longer duration of dosing ( $\geq$ 1 month). They also suggested that when considering 4 $\beta$ -OHC as a metric, one should take into account assay precision, the anticipated magnitude of the effect, and the feasibility of dosing beyond 2 weeks. In addition, the 4 $\beta$ -OHC metric needs to be normalized with the corresponding cholesterol plasma level in the same subject. Goodenough et al. (2011) also reported that a CYP3A inducer (rifampin) increased the 4 $\beta$ -OHC concentration significantly, while the decrease of 4 $\beta$ -OHC level was moderate when a CYP3A inhibitor (ketocconazole) was used. In this work, 14 subjects were dosed with rifampin and 13 subjects were treated with ketoconazole. Their results indicated that an average increase of 151.4% in 4 $\beta$ -OHC over

baseline was observed (range from 52.4 to 350.6% increase in 4 $\beta$ -OHC) for the group treated with rifampin, while an average decrease over predose values of 16.7% (range from -38.2 to +9.0% change in 4 $\beta$ -OHC) was observed for the group dosed with ketoconazole. Arrhén et al. (2013) compared plasma 4 $\beta$ -OHC: cholesterol with urinary 6 $\beta$ -HC: cortisol as markers of cytochrome P450 3A4 activity before and after treatment with rifampicin for 2 weeks. Their results showed that plasma 4 $\beta$ -OHC: cholesterol gave similar induction ratios to urinary 6 $\beta$ -HC: cortisol. Björkhem et al. (2013) carried out a study for the comparison of endogenous 4 $\beta$ -OHC with midazolam as markers for CYP3A4 induction by rifampicin. This study compared the endogenous 4 $\beta$ -OHC: cholesterol ratio with the oral midazolam clearance as markers for CYP3A4 induction. Their results indicated that the 4 $\beta$ -OHC: cholesterol ratio was comparable with midazolam clearance as a marker of CYP3A4 induction. Major advantages of the former include that it is endogenous and no drug must be given; with the latter, it can be used to record rapid changes in CYP3A activity such as inhibition.

Other than the studies described earlier for the use of 4 $\beta$ -OHC as an endogenous biomarker for CYP3A activity in humans, a recent study also reported (Li et al., 2014) the use of 4 $\beta$ -OHC as an endogenous biomarker of CYP3A activity in cynomolgus monkeys. Following multiple oral administration of rifampicin at 15 mg/kg/day in cynomolgus monkeys, the mean serum 4 $\beta$ -OHC levels increased fourfold from the baseline. The mean concentration ratios of 4 $\beta$ -OHC to cholesterol increased fivefold. The data suggested that 4 $\beta$ -OHC formation from cholesterol metabolism was induced by rifampicin treatment in monkeys. This observation correlated with the metabolism of midazolam monitored in the same study. The serum exposure of midazolam was markedly decreased by approximately 95%, confirming the induction of CYP3A catalytic activity by rifampicin treatment in monkeys. The results suggested that 4 $\beta$ -OHC could be used as an endogenous biomarker to identify strong CYP3A inducers in cynomolgus monkeys, which may help to evaluate DDI potential of drug candidates in pre-clinical settings.

### **19.2.2 Quantitation of 4 $\beta$ -OHC in Human and Animal Species**

As discussed in the last section, the use of 4 $\beta$ -OHC as an endogenous biomarker for CYP3A activity has attracted an increased interest and has been used in the clinical trials in support of drug development. Therefore, it was important to have a robust method for the accurate determination of 4 $\beta$ -OHC in human and animal species. The analysis of 4 $\beta$ -OHC was reported using either gas

chromatography–mass spectrometry (GC–MS) or liquid chromatography–tandem mass spectrometry (LC–MS/MS).

The GC–MS methods (Bodin et al., 2001; Olof, 1995) used for the analysis of 4 $\beta$ -OHC required large sample volume, long sample preparation time (overnight for saponification and derivatization), and long run time (longer than 25 min), which was not the best choice for high-throughput sample analysis. Therefore, researchers have explored the opportunities of using LC–MS/MS for the quantitation of 4 $\beta$ -OHC. This section will mainly discuss the quantitation of 4 $\beta$ -OHC by LC–MS/MS.

The recent published LC–MS/MS methods utilized various MS ionization techniques and sample preparation procedures and resulted in various assay sensitivities. Honda et al. reported the first LC–MS/MS method (Honda et al., 2009) for the analysis of 4 $\beta$ -OHC and other six oxysterols in human serum and rat liver microsomes. This method utilized electrospray ionization (ESI) and derivatized 4 $\beta$ -OHC into a picolinyl ester to enhance the ionization efficiency. However, this method had a very long run time of 40 min, which was not conducive for routine sample analysis. In 2011, the use of atmospheric pressure photoionization (APPI)–LC–MS/MS for the analysis of 4 $\beta$ -OHC in human plasma was reported (Van de Merbel et al., 2011). This method was based on alkaline hydrolysis (saponification) to convert esterified 4 $\beta$ -OHC to free 4 $\beta$ -OHC, followed by extraction from plasma by hexane and purification of the hexane extract by normal-phase solid-phase extraction (SPE). The analyte was chromatographically separated from endogenous isobaric oxysterols and excessive cholesterol by a 16-min reversed-phase gradient on a C18 column; detection was performed by APPI–tandem mass spectrometry (MS/MS) in the positive ion mode, using toluene as a dopant. Cholesterol also needs to be separated from 4 $\beta$ -OHC due to the potential in-source oxidation of cholesterol to 4 $\beta$ -OHC and also to minimize the ionization competition between 4 $\beta$ -OHC and cholesterol, which has a concentration much higher than that of 4 $\beta$ -OHC. The advantage of this method is no requirement of derivatization. However, it required large sample volume (400  $\mu$ L) to achieve the needed sensitivity. APPI, a much less used source, is not easily available in most of the bioanalytical laboratories, and it also requires a post-column addition of reagent to facilitate the photo ionization. Goodenough et al. published an assay (Goodenough et al., 2011) in 2011 for the analysis of 4 $\beta$ -OHC using LC/ESI–MS/MS with a lower limit of quantification established at 2 ng/mL using 50  $\mu$ L of plasma. The entire sample preparation scheme including saponification and derivatization of 4 $\beta$ -OHC to the corresponding dipicolinyl ester was completed in less than 8 h using an automated sample preparation scheme, enabling higher-throughput capabilities.

Chromatographic resolution of 4 $\beta$ -OHC from 4 $\alpha$ -OHC and other endogenous isobaric species was achieved in 11 min using an isocratic elution on a C18 column. This assay used a stable-isotope labeled-(SIL) analogue, d7-4 $\beta$ -OHC, as a surrogate analyte that was measured in the standard curve and quality control samples prepared in plasma. A second SIL analog, d4-4 $\beta$ -OHC, was used as the internal standard. Although this approach mitigated the challenge posed by endogenous level in the blank matrix, it did require two versions of stable-labeled analytes and a more complicated calculation scheme. This type of approach is costly and is more suitable for the relative measurement (e.g., comparing biomarker level changes from placebo to dosed subjects) (Jian et al., 2010, 2013). Additionally, it was reported that d7-4 $\beta$ -OHC was not an ideal surrogate analyte because it displayed considerable isotope effect during chromatographic separation as indicated by a 2–3 s shorter retention time compared with 4 $\beta$ -OHC (Xu et al., 2013). Xu et al. recently reported (Xu et al., 2013) another LC/ESI-MS/MS assay for the analysis of 4 $\beta$ -OHC and cholesterol in chimeric mice with humanized livers and human plasma using human serum albumin (HSA) in phosphate-buffered saline (PBS) solution as surrogate matrix for the preparation of both calibration standard and QC samples. The purpose of their study was to investigate whether or not the newer chimeric FRG mice with humanized livers (when the Fah(–/–) mice were crossed with Rag-2(–/–) and interleukin-2 receptor subunit gamma (–/–) mice, a triple mutant mouse was produced (FRG)) model might predict better human metabolism and disposition since the hepatocytes from the repopulated FRG mouse liver were indistinguishable from normal human hepatocytes from readouts using standard drug metabolism assays. The assay used only 5  $\mu$ L of sample to reach an LLOQ of 5 ng/mL for 4 $\beta$ -OHC and 50  $\mu$ g/mL for cholesterol with an analytical run time of 16 min. The assay was qualified to analyze small sets of research samples, but was not optimized for routine sample analysis from clinical studies. In 2014, Huang et al. (2014) reported a validated LC/ESI-MS/MS for the analysis of 4 $\beta$ -OHC in human plasma. In this assay, water was used as surrogate matrix for the preparation of calibration standard, and authentic human plasma was used as matrix for the preparation of QC samples to mimic the incurred study samples. In the absence of an authentic 4 $\alpha$ -OHC standard at the time of this study, the paper also described an alternative selectivity test strategy to confirm the separation between 4 $\beta$ -OHC and 4 $\alpha$ -OHC. Although the author's lab attempted to synthesize pure 4 $\alpha$ -OHC as a marker, only a mixture of 4 $\alpha$ -OHC and its isomers (including 4 $\beta$ -OHC) was obtained. It was suspected that the major peak (about 80% HPLC peak area) from the synthesized mixture was 4 $\alpha$ -OHC, but there was not enough characterization data

to prove this directly. In order to confirm the hypothesis, an indirect approach was used, briefly summarized as follows: the synthesized mixture was derivatized and analyzed following a published GC-MS method (Bodin et al., 2001); the retention times of 4 $\alpha$ -OHC and 4 $\beta$ -OHC reported in that paper were compared with the retention times of 4 $\alpha$ -OHC and 4 $\beta$ -OHC obtained from the synthesized mixture. The results clearly confirmed the hypothesis since the retention times of 4 $\alpha$ -OHC and 4 $\beta$ -OHC in the published paper and from the synthesized mixture matched very well. The 4 $\alpha$ -OHC in the synthesized mixture was used as reference to confirm the separation between 4 $\beta$ -OHC and 4 $\alpha$ -OHC in the final LC-MS method. After the assay was validated, a commercially available 4 $\alpha$ -OHC standard was obtained and the hypothesis was confirmed directly. The calibration curve range was 5–500 ng/mL with an analytical run time of 12 min. The validated assay was successfully applied to a phase I clinical study for the measurement of 4 $\beta$ -OHC in human plasma.

Although no published assays indicated that antioxidant was needed during sample storage, Diczfalusi et al. reported that there was potential risk of *in vitro* autoxidation of cholesterol to 4 $\beta$ -OHC if the samples are stored for an extended period of time at –20°C, while 4 $\alpha$ -OHC and 4 $\beta$ -OHC were stable for a few years if samples are stored at –70°C (Diczfalusy et al., 2011). They had analyzed samples stored for longer periods of time (and frozen/thawed a few times), where the concentrations of 4 $\alpha$ -OHC had increased up to 100 ng/mL and were closely correlated with the increased concentrations of 4 $\beta$ -OHC. They believed that this was due to nonenzymatic formation of both 4 $\alpha$ - and 4 $\beta$ -OHC during uncontrolled storage conditions. Based on this finding, monitoring the concentration changes of 4 $\alpha$ -OHC might be useful as surrogate for elucidating the potential *in vitro* autoxidation of cholesterol to 4 $\beta$ -OHC for samples that have been stored at –20°C for more than 1 year.

More recently, an industrial white paper was published to address bioanalytical best practice and criteria for the LC-MS/MS analysis of 4 $\beta$ -OHC in plasma (Aubry et al., 2016). In this paper, it was recommended that assay precision and reproducibility are the key assay attributes in assessing CYP3A4 activity, and to reduce analytical variability, samples from a single subject should be analyzed together to facilitate interpretation of study results.

### 19.2.3 24S-OHC and 27-OHC as Biomarkers

In addition to 4 $\beta$ -OHC, 24S-OHC and 27-OHC have also been reported as biomarkers. 24S-OHC is the most abundant hydroxycholesterol in the brain and is the primary transport form of cholesterol from the central nervous system into the blood, with smaller amounts

being eliminated through cerebrospinal fluid (CSF). The cytochrome P450 responsible for formation of 24S-OHC, cholesterol 24S-hydroxylase (CYP46), is almost exclusively located in the brain in humans, and most or all of the 24S-OHC present in human circulation is thus derived from the brain. There is a continuous flux of 24S-OHC across the blood–brain barrier into the circulation, and evidence has been provided that this flux is of importance for the homeostasis of brain cholesterol. Thus, the levels of 24S-OHC in the circulation can be used as a marker for the turnover of cholesterol in the brain (Björkhem et al., 1998). 24S-OHC is often expressed as a ratio to 27-OHC. Serum or plasma concentration ratios of the brain-specific cholesterol metabolite 24S-OHC to the peripheral metabolite 27-OHC may be useful surrogate markers for neurodegenerative diseases including Alzheimer's disease, Huntington's disease, human immunodeficiency virus (HIV)-associated neurocognitive disorders, and multiple sclerosis (Lütjohann and von Bergmann, 2003). Compared with other available biomarkers in CSF, such as total Tau, phospho-Tau, and  $\beta$ -(42) amyloid, the 24S-OHC appears to be the most sensitive biomarker in the evaluation of patients with cognitive impairment disease (Leoni, 2009). The levels of 24S-OHC in the circulation reflect the balance between cerebral production and hepatic degradation and may provide some information concerning cholesterol homeostasis in the brain in connection with neurological and neurodegenerative diseases.

27-OHC is formed primarily in the periphery by the enzymatic action of CYP 27A1 on cholesterol (Fakheri and Javitt, 2012). The levels of 27-OHC in the circulation seem to be elevated more often in a population of patients with atherosclerosis than in control subjects, possibly reflecting an activated defense mechanism in the patients. In some cases, the levels of 27-OHC may be elevated by a reduced metabolism for various reasons (Björkhem and Diczfalussy, 2002). In addition, 27-OHC promotes proliferation and metastasis in estrogen receptor-dependent ( $ER^+$ ) breast cancers, and 27-OHC level has been shown to be elevated in  $ER^+$  breast cancer patients (Nelson et al., 2013; Wu et al., 2013).

#### 19.2.4 Quantitation of 24S-OHC and 27-OHC

There were several publications dedicated to the quantification of 24S-OHC and 27-OHC in human plasma or serum (Burkard et al., 2004; Venkata et al., 2014; Sugimoto et al., 2015). Burkard et al. developed a sensitive and specific LC–MS method for the quantification of 24S-OHC and 27-OHC in human plasma (Burkard et al., 2004). In contrast to currently available procedures based on GC–MS, this methodology offered the advantage that the procedure of time-consuming derivatization was not needed. After saponification, SPE, and

HPLC separation, detection by MS using atmospheric pressure chemical ionization (APCI) was performed. The standard curves were linear throughout the calibration range of 40–400 ng/mL for both oxysterols. The standard curves were plotted as the peak area ratio of the respective compound to the internal standard versus the concentration and then corrected for endogenous oxysterols in the human plasma by subtracting blank peak area ratios. Within-day and between-day coefficients of variation were less than 9%, and the recoveries ranged between 98 and 103%. Venkata et al. (2014) developed a sensitive LC–MS/MS method in ESI mode using simple liquid–liquid extraction (LLE) of 0.5 mL of sample volume for simultaneous quantitative determination of free 24S-OHC and 27-OHC in serum. The calibration curve range was established at 10–1000 ng/mL for both analytes using blank serum samples for background correction. Sugimoto et al. (2015) developed a highly sensitive and specific LC–MS/MS method with APCI to determine 24S-OHC in human plasma without any derivatization steps. PBS containing 1% Tween 80 was used as the surrogate matrix for preparation of calibration curves and quality control samples. To confirm the suitability of surrogate matrix approach, the QC samples in human plasma (unspiked and additionally spiked at LQC, MQC, HQC) were assayed. The results showed the robustness of this analytical method for 24S-OHC in human plasma and ascertained the suitability for the use of PBS as a surrogate matrix. The saponification process to convert esterified 24S-OHC to free sterol was optimized, followed by LLE using hexane. Chromatographic separation of 24S-OHC from other isobaric endogenous oxysterols was successfully achieved with gradient elution on an L-column2 ODS (2  $\mu$ m, 2.1 mm id  $\times$  150 mm). This assay had a calibration curve range of 1–100 ng/mL using 200  $\mu$ L of sample volume with acceptable intra- and inter-day precision and accuracy. The potential risk of *in vitro* formation of 24S-OHC by oxidation from endogenous cholesterol in human plasma was found to be negligible. This method was successfully applied to quantify the plasma concentrations of 24S-OHC in male and female volunteers in a clinical trial.

### 19.3 Cortisol and 6 $\beta$ -Hydroxycortisol (6 $\beta$ -HC) as Biomarkers and Their Quantitation

#### 19.3.1 Cortisol and 6 $\beta$ -HC as Biomarkers

Cortisol is a steroid hormone, of the glucocorticoid class of hormones, and is produced in humans by the zona fasciculata of the adrenal cortex within the adrenal gland. It is released in response to stress and low blood-glucose

concentration. It functions to increase blood sugar through gluconeogenesis, to suppress the immune system, and to aid in the metabolism of fat, protein, and carbohydrate. It also decreases bone formation (Chyun et al., 1984). Levine et al. reported (2007) that cortisol circulated in the blood in both free and bound forms. In plasma, cortisol is predominantly bound to corticosteroid-binding globulin, with a small amount bound loosely to albumin, and the remainder is free. The remaining free cortisol molecule is lipophilic and, with a low molecular weight, passes from capillaries into tissues mainly by passive diffusion. The biologically active free fraction comprises only 2–5% of the total hormone concentration. Most of cortisol is excreted into urine as tetrahydrocortisol metabolites conjugated glucuronides and sulfates, and only about 3% occurs as the native form (Turpeinen and Hämäläinen, 2013). Cortisol is metabolized to 6 $\beta$ -HC by CYP 3A4, an enzyme implicated in the critical epoxidation reactions of aflatoxin and certain polycyclic aromatic hydrocarbon (Joellenbeck et al., 1992).

Cortisol has long been used in human psychobiological studies as a biological marker of stress, anxiety, and depression (Levine et al., 2007). Cortisol is involved in various physiological and pathological functions and is measured in different matrices including plasma, serum, saliva, urine, and even hair as a biomarker of various conditions. Cortisol levels in the body fluids are characterized by circadian rhythm with a morning maximum, declining levels throughout the daytime, a period of low concentration around midnight, and a rise after the first few hours of sleep (Gatti et al., 2009). Cortisol level measurement is used in the assessment of adrenal, pituitary, and hypothalamic functions and is especially important in the diagnoses of Cushing's syndrome and Addison's disease. Total serum cortisol, 24-h urinary free cortisol, and salivary free cortisol measurements are utilized in cortisol metabolism study for hyper- and hypo-cortisolism identification (Gatti et al., 2009).

Salivary cortisol is routinely used as biomarker of psychological stress and related mental or physical diseases. Most studies consider salivary cortisol levels a reliable measure of hypothalamus–pituitary–adrenal axis (HPAA) adaptation to stress (Hellhammer et al., 2009). Cortisol in saliva has been suggested to be a good biomarker for evaluation of stress objectively because it can be collected easily in a stress-free manner without medical supervision, it allows direct measurements, and it is strongly correlated with plasma cortisol levels. However, the cortisol level in saliva is lower than those in serum and urine (Kataoka et al., 2007). Alternatively, measurement of human hair has been reported as biomarker of systemic exposure. Hair grows approximately 1 cm per month (range 0.6–1.4 cm) and a hair sample of 2–3 cm reflects average hormone levels over the previous

2–3 months. Hence, unlike other matrices, hair cortisol may represent long-term exposure to the hormone (Sauvé et al., 2007).

The quantification of cortisol and 6 $\beta$ -HC and its ratio in human urine is utilized as a biomarker of CYP3A activity (Remer et al., 2008; Lutz et al., 2010). Although the ideal CYP3A activity probe is not yet identified, in drug development, 6 $\beta$ -HC/cortisol can be used to indicate whether a drug is a CYP3A4 inducer or inhibitor. In general, an increase in 6 $\beta$ -HC/cortisol indicates that the drug may be a potential CYP3A4 inducer, and a decrease in the ratio indicates that the drug may be a CYP3A4 inhibitor (Barrett et al., 2005).

In recent years, sensitive LC–MS/MS assays have been developed to measure cortisol in saliva, serum, urine and hair. LC–MS/MS technology coupled with an effective sample extraction procedure facilitates reliable quantification of serum, salivary, and urinary cortisol levels. In general, LC–MS/MS assays are superior to immunoassays (IAs) in accuracy and selectivity. Decreasing costs of the LC–MS/MS equipment, new user-friendly reagent kits, and analytical protocols along with the use of convenient sample-handling devices make LC–MS/MS a very competitive choice for cortisol analysis. A summary of some of the published LC–MS/MS assays is listed in Table 19.3.

### 19.3.2 Measurement of Cortisol and 6 $\beta$ -HC

#### 19.3.2.1 Measurement of Cortisol in Serum

The most widely used methods for serum cortisol assay are based on IA, which can be easily automated. More than 30 different methods are available as manual kits or on automated platforms. The label in these assays can be a radioactive isotope (radioimmunoassay (RIA)), an enzyme (enzyme immunoassay (EIA)), a fluorophore (fluorescent immunoassay (FIA)), or a luminescent label (luminescent immunoassay (LIA)). The between-assay agreements of these methods are usually unsatisfactory. When compared with tandem mass spectrometry, many IAs show both over- and underestimations of true cortisol concentrations, which may lead to erroneous clinical decisions (Turpeinen and Hämäläinen, 2013; Briegel et al., 2009). Mass spectrometer assays are gaining more popularity and are a method of choice. A selective method with SPE and low sample volume has been reported for quantitation of cortisol in serum and plasma samples (Ray et al., 2011). Compounds that potentially interfere with tandem mass spectrometry detection could be isobars of the target analyte (e.g., cortisone/prednisolone, cortisol/tetrahydroprednisolone, testosterone/dehydroepiandrosterone (DHEA), cortisol/fenofibrate), isotopic ions of the molecules with lower  $m/z$ , or adducts of the impurities, which are isobaric to the analyte or the internal standard. Common types of interference among

**Table 19.3** Published LC-MS assays for the quantitation of cortisol and 6 $\beta$ -HC.

Analyte	Platform and author	Sample matrix	Sample amount	Curve range
Cortisol and 6 $\beta$ -HC	LC-ESI-MS/MS Lutz et al. (2010)	Human urine	300 $\mu$ L	0.5–200 ng/mL for Cortisol 2.0–800 ng/mL for 6 $\beta$ -HC
Cortisol and 6 $\beta$ -HC	LC-ESI-MS/MS Barrett et al. (2005)	Human urine	100 $\mu$ L	0.2–100 ng/mL for Cortisol 1.0–500 ng/mL for 6 $\beta$ -HC
Cortisol	LC-ESI-MS/MS Chen et al. (2014)	Human urine	100 $\mu$ L	0.5–250 ng/mL
Cortisol	LC-ESI-MS/MS Chen et al. (2014)	Human hair	20 mg	1.25–100 pg/mg
Cortisol	LC-ESI-MS Kataoka et al. (2007)	Human saliva	100–200 $\mu$ L	50–2000 pg/mL
Cortisol	LC-ESI-MS/MS Lee et al. (2010)	Human saliva	1000 $\mu$ L	0.2–25 ng/mL

steroids are +2 isotopes of their unsaturated analogs (cortisone with cortisol, estrone with estradiol, prednisone with prednisolone, prednisolone with cortisol, cortisol with fenofibrate, testosterone and DHEA with dihydrotestosterone (DHT), etc.). Interestingly, the interference of fenofibrate with cortisol illustrates that with MS/MS detection the interfering substances are not necessarily chemically or structurally related to the analyte of interest (Kushnir et al., 2011; Meikle et al., 2003). Fenofibrate presents an interesting analytical challenge for cortisol because not only is the retention time of this compound similar to the retention time of cortisol under some HPLC conditions on reversed-phase (C18) LC columns, but also its molecular mass is only 2 Da lower than that of cortisol. Fenofibrate structure contains a chlorine atom; hence, its mass spectrum contains a strong (about 30%) isotope peak 2 Da higher than its nominal molecular mass. This isotope is an isobar of cortisol and produces the same molecular ion as the one selected for the MS/MS analysis. Furthermore, product mass spectra of the fenofibrate molecular ion also produce a strong fragment at *m/z* 121, a potentially very troubling interference. However, fenofibrate does not generate an *m/z* 97 product ion peak, which is specific for cortisol. By using the *m/z* 97 ion for quantitation (for those samples whose ratio of the 121–97 peak is high compared with the standard), interference from fenofibrate can be eliminated (Meikle et al., 2003).

Cortisol exists in plasma in free- and protein-bound forms. Measuring total cortisol might be easier and may cover most of the clinical studies, but measuring free cortisol may be more accurate to reflect certain conditions. Methods for assaying serum free cortisol

are complex, time consuming, and expensive (including ultrafiltration, equilibrium dialysis, steady-state gel filtration, etc.) (Gatti et al., 2009).

### 19.3.2.2 Measurement of Cortisol and 6 $\beta$ -HC in Urine

CYP3A enzyme catalyzes the C-6 oxidation of cortisol to form 6 $\beta$ -HC. The metabolite 6 $\beta$ -HC is then excreted as an unconjugated form in urine (Furuta et al., 2004). The metabolic ratio of the concentration of 6 $\beta$ -HC to cortisol in human urine had been proposed as an endogenous marker for CYP3A activity (Lutz et al., 2010). Cortisol and its metabolites can be measured by different detection methods including thin-layer chromatography (TLC), IA, GC-MS, and LC-MS/MS. IA is usually used for determination of the steroid hormones in body fluids in the clinical laboratory due to its simplicity and superior sensitivity. Despite the many advantages, these methods could lead to falsely elevated values due to poor antibody specificity (Zhai et al., 2015). The latest generation of GC-MS and LC-MS/MS techniques is superior to IA regarding assay selectivity and specificity, providing a good linearity even down to low concentrations. Since LC-MS/MS can provide measurement with high sensitivity and high specificity as well as high throughput, many diagnostic laboratories are adopting this new technology (Krone et al., 2011).

A GC-MS profiling of steroids in urine of patients with acute intermittent porphyria has been reported, and the results revealed a basal decrease of steroid 5 $\alpha$ -reductase activity in the liver, related to malnutrition and hepatic energy misbalance associated with the disease (Casals et al., 2013). Several LC-MS/MS methods for the

detection of cortisol and  $6\beta$ -HC have also been reported. A sensitive method for quantitation of urinary  $6\beta$ -HC and cortisol using online SPE and LC–MS/MS was developed and validated (Barrett et al., 2005). In that method, human urine samples were injected directly onto an online SPE apparatus, followed by HPLC separation and ESI triple-quadrupole LC–MS/MS detection. This method was able to determine the urinary  $6\beta$ -HC to cortisol ratio with adequate sensitivity and reproducibility. In addition, the method was fully automated with only minor operator intervention. In general, boric acid is recommended to be used as a preservative for urinary cortisol analysis in clinical practice to reduce bacterial action for samples not analyzed within 2 h of collection. This method recommended that boric acid be added whenever urine specimens are to be stored at room temperature for longer than 8 h. A new UPLC–MS method (Han et al., 2011) was developed for the precise and accurate quantitation of  $6\beta$ -HC and free cortisol in human urine, using high-resolution ESI-QTOF MS with full mass detection. With the optimized LC mobile phase and the sample preparation procedure, femtomolar sensitivities on column were achieved, and good precision and accuracy were demonstrated. The method has been successfully applied to the determination of the metabolic  $6\beta$ -HC and cortisol ratios on a cohort of premenopausal women. This proposed method provides an alternative approach to LC–MS/MS for reliable quantitation of urinary  $6\beta$ -HC and free cortisol. Another study described a rapid, simple, and reproducible LC–APCI–MS/MS method for the simultaneous determination of urinary  $6\beta$ -HC and cortisol (Tang et al., 2000). The sample preparation procedures of LLE and SPE were compared. This method successfully detected the induction of CYP3A in monkeys treated orally with two clinically relevant inducers and demonstrated that the Rhesus monkey could be used as an animal model for evaluating the potential for induction of CYP3A in man. This method may be applicable in early phases of drug development for the screen of drug candidates as potential CYP3A inducers.

#### 19.3.2.3 Measurement of Cortisol in Saliva and Hair

Salivary cortisol is a useful tool for detection of hypercortisolism and fluctuation of cortisol production. Salivary cortisol is also used to monitor glucocorticoid treatment of patients with congenital adrenal hyperplasia and may be useful for monitoring of hydrocortisone replacement therapy. An advantage of salivary cortisol testing is the easy, noninvasive sample collection. By using the Salivette polyester swab device, which does not adsorb steroids, patients can collect saliva samples at home or in hospital wards under stress-free conditions.

Salivary samples can be transferred to the laboratory during the following days. Salivary cortisol is stable at room temperature for 1–2 days and at refrigerator temperature for a week (Turpeinen and Hämäläinen, 2013). A highly sensitive and specific analytical method was used to determine the very low concentrations of cortisol in saliva (Lee et al., 2010). In this method, the control matrix was prepared by stripping steroids from saliva using activated charcoal. Saliva pooled from healthy volunteers was stirred with 1 g of activated charcoal overnight and then centrifuged at 4000 rpm for 15 min. The supernatant was used as the steroid-free saliva, in which cortisol was not detected. Working calibrators were prepared by dilution of the stock solutions in the steroid-free saliva. Calibration standards and quality control samples were stored at  $-20^{\circ}\text{C}$  until analysis. The extraction was carried out using Oasis<sup>®</sup> HLB SPE columns. Another simple, rapid, and sensitive method for the determination of cortisol levels in human saliva has been reported (Kataoka et al., 2007). Standard cortisol was added to pooled saliva sample at the concentration of 0, 50, 100, 200, 500, 1000, and 2000 pg/mL, and a calibration curve was constructed. In this method, cortisol was analyzed by online in-tube solid-phase microextraction (SPME) coupled with LC–MS. Dynamic desorption of cortisol from the capillary could be readily achieved by switching the six-port valve. The desorbed cortisol was transported to the LC column by mobile-phase flow for MS detection.

Until a decade ago, cortisol had predominantly been measured in saliva, serum, or urine, which allows analyzing the dynamics of cortisol production for up to 24 h. The analysis of hair cortisol concentrations is a promising new tool for the assessment of long-term cortisol level. With the recent advancement in sample preparation and LC–MS/MS, measurement of cortisol and other steroids in scalp hair has become possible (Nope et al., 2015; Staufenbiel et al., 2015). For example, an enhanced assay for simultaneous measurements of cortisol in hair and urine samples have been reported (Chen et al., 2014). In this method, after being washed twice with 1 mL of methanol, hair strands were milled by a ball mill (MM 400, Retsch, Germany) for 4 min at 30 Hz. 20 mg of hair powder was incubated at  $40^{\circ}\text{C}$  for 14 h in 1 mL of methanol in the presence of 2.5 ng of d4-cortisol. The incubation solution was separated by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a dry tube and evaporated to dryness by  $\text{N}_2$  at  $50^{\circ}\text{C}$ . The dry residue was resuspended in 50  $\mu\text{L}$  of methanol and then diluted by 1 mL of water. The diluted solution was extracted using an SPE C18 column, activated sequentially by 3 mL of methanol and 3 mL of water. The deposit on the SPE C18 column was rinsed with 3 mL of water, dried for 30 min, and eluted by 1 mL of methanol. The eluent was

evaporated to dryness in pure N<sub>2</sub> at 50°C and redissolved in 50 µL of mobile phase for LC-MS/MS analysis. LOD and LLOQ were 0.5 and 1.25 pg/mg, respectively, for hair steroids. These assays show that analysis of cortisol in hair is a new tool for assessment of steroids.

## 19.4 Summary

It was proposed that 4β-OHC could be used as a biomarker for CYP3A4/5 activity with potential use in drug development. Based on literature data, it is relatively more sensitive to use 4β-OHC to evaluate CYP3A induction than to evaluate CYP3A inhibition. The long half-life (about 17 days) of 4β-OHC results in small variations within subjects in plasma concentration and is an advantage when 4β-OHC is used as a marker for CYP3A activity during steady-state conditions. In contrast, the long half-life excludes 4β-OHC as a marker for measuring rapid changes in CYP3A activity. Other oxysterols such as 24S-OHC and 27-OHC have been also reported as potential biomarkers for neurodegenerative diseases

including Alzheimer's disease, Huntington's disease, HIV-associated neurocognitive disorders, and multiple sclerosis. 4β-OHC, 24S-OHC, and 27-OHC have been analyzed by different LC-MS/MS methods with different sample preparation procedures and instrument types. Since each method has its own advantages and disadvantages, one may choose individual methods based on the study purpose and the resources available in the bioanalytical laboratory.

Cortisol determinations are used in the assessment of overproduction in Cushing's syndrome or hypo-cortisolism in Addison's disease. In addition, cortisol has been used as a biological biomarker in human psychobiological studies for stress, anxiety, and depression. Measurement of cortisol is not limited to serum/plasma but has been conducted in different matrices including saliva, urine, and hair. Especially 6β-HC and cortisol ratio has been used as a biomarker for CYP3A4 activity in urine samples. With advances in instrumentation and chromatographic methods, cortisol in different matrices can be measured with high specificity, sensitivity, selectivity, and reliability using LC-MS/MS methods.

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## 20

### Bile Acids as Biomarkers

Clara John<sup>1</sup>, Philipp Werner<sup>2</sup>, Joerg Heeren<sup>1</sup>, and Markus Fischer<sup>2</sup>

<sup>1</sup> University Medical Center Hamburg-Eppendorf (UKE), Institute of Biochemistry and Molecular Cell Biology, Hamburg, Germany

<sup>2</sup> University of Hamburg, Hamburg School of Food Science, Hamburg, Germany

#### 20.1 Introduction

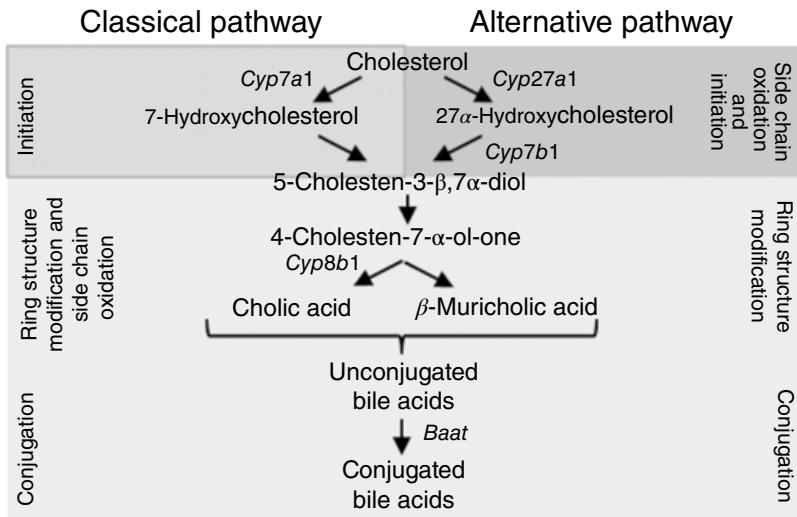
Bile acids (BAs) are amphipathic steroids that are generated by degradation and modification of cholesterol (Figure 20.1). Together with cholesterol, phospholipids, and bilirubin, BAs are the principal constituents of bile. In humans the hepatic BA synthesis pathway converts daily 500 mg of cholesterol to BA under physiological conditions. This production rate accounts for 90% of metabolized cholesterol, whereas the remaining 10% serve as a precursor for steroid hormone—or vitamin D synthesis (Russell, 2003).

BA synthesis is conducted either by the classical or the alternative synthesis pathway and involves 17 different enzymes in total. Both pathways consist of an initiation step, modification of the steroid backbone, oxidation reactions, and finally transformation of unconjugated BA (UBA) to conjugated BA (CBA) (Figure 20.1). Within the conjugation procedure, bile acid-CoA:amino acid *N*-acyltransferase (BAAT) catalyzes the addition of glycine or taurine to a UBA. In humans, predominantly glycine is used for conjugation, whereas in mice taurine-conjugated species are the most abundant ones. Hence, the biochemical characteristics of BA derived from human and mice are not totally comparable. However, consumption of a *Western style* diet enriched with taurine leads to a shift toward taurine-conjugated BA also in humans (Hardison, 1978).

In hepatocytes, BA synthesis is under a feedback transcriptional control by ligand-activated nuclear receptors that are regulated in dependence on the concentration of cholesterol, its derivatives, and BA. In this line, high intracellular concentrations of cholesterol result in a higher abundance of hydroxylated cholesterol derivatives such as 25-hydroxycholesterol. These metabolites induce the activation of liver X receptor (LXR) that in turn enhances the expression of the ATP-binding

cassette subfamily G members 5 and 8 (*Abcg5* and *Abcg8*). These transporters located in the apical membrane of hepatocytes mediate biliary cholesterol efflux. Moreover, LXR controls the expression of *cytochrome p4507A1* (*CYP7A1*) and the rate-limiting enzyme of the classical BA synthesis pathway. Thus, LXR activation drives direct biliary secretion of cholesterol as well as the conversion of excess cholesterol into BA.

On the other hand, *farnesoid X receptor* (*FXR; Nr1h4*) works as a negative regulator of BA synthesis (Parks et al., 1999). FXR is activated by high intracellular concentrations of specific BA, causing an increased expression of *small heterodimer partner* (SHP; *Nr0b2*). Subsequently, the interaction of SHP with the nuclear receptor *liver receptor homolog-1* (LRH-1; *NR5A2*) results in a reduced expression of *Cyp7a1*. Moreover FXR activation induces an accelerated biliary BA transport by stimulating the expression of *bile salt export pump* (*BSEP; Abcb11*) (Stieger et al., 1992). FXR activation is dependent on the affinity of a distinct BA species toward its binding site (Kuipers et al., 2014a, 2014b). The most efficient agonist is chenodeoxycholic acid (CDCA) followed by deoxycholic acid (DCA), lithocholic acid (LCA), and cholic acid (CA) (Makishima et al., 1999; Parks et al., 1999). On the other hand, very hydrophilic BA, such as tauro- $\beta$ -muricholic acid (T- $\beta$ -MCA), even antagonizes FXR action (Sayin et al., 2013). Regarding the hydrophobicity, it has been described that more hydrophilic BA stimulates BA efflux and inhibit intestinal uptake of dietary cholesterol (Wang et al., 2003; Thomas et al., 2008). Moreover, a deficiency in 12 $\alpha$ -hydroxylated BA in *Cyp8b1*<sup>-/-</sup>-mice is associated with the development of a diabetic phenotype in an FXR-dependent manner (Haeusler et al., 2012), underlining the importance of specific BA species for the activation of ligand-activated nuclear receptors regulating steroid, lipid, and general energy homeostasis.



**Figure 20.1** Simplified scheme of bile acid synthesis via the classical or alternative pathway including rate-limiting enzymes (in italic).

The classical function of BA is the emulsification of dietary lipids in order to facilitate lipid uptake. Therefore, in a postprandial situation, BAs are released in the intestinal lumen, thereby increasing the concentration in BA not only in the gut but also in the liver and the systemic circulation (Ho, 1976; Engelking et al., 1980; Everson, 1987). Based on the composition of the gut microbiota, BAs undergo several deconjugation, dehydroxylation, and dehydrogenation reactions. Hence, not only the chemical diversity of the so produced secondary BA (SBA) but also their biological activity is multiplied (Sayin et al., 2013; Ridlon et al., 2014). Nevertheless, BAs themselves have also been described to affect the composition of the intestinal microflora (Hofmann and Eckmann, 2006; Inagaki et al., 2006), whereas the latter is also shaped by the type and amount of the diet (Wu et al., 2011; Ridaura et al., 2013). In this regard, obesity-induced alterations of the gut microbiome are associated with elevated concentrations of DCA, thereby increasing the risk colorectal as well as hepatocellular cancer (Schwabe and Jobin, 2013; Yoshimoto et al., 2013).

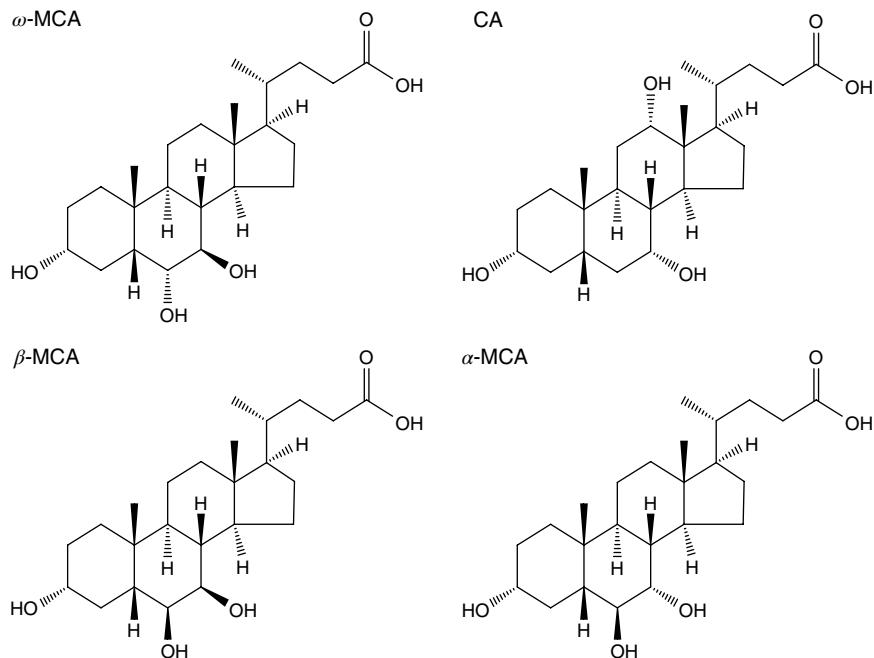
In the course of the enterohepatic circulation, the majority of BAs are shuttled back to the liver and only the minor part (~5%) is excreted. While CBA are taken up predominantly via the *apical sodium-dependent bile acid transporter* (ASBT, *Slc10a2*) into enterocytes of the terminal ileum, UBA enters the cells by passive diffusion. In enterocytes, BAs stimulate FXR-mediated negative feedback regulation of hepatic BA synthesis via an endocrine route. Intestinal FXR activation results in the release of *fibroblast growth factor 15/19* (FGF15/19) that in turn stimulates a signaling pathway, causing the inhibition of BA synthesis in hepatocytes. On their route back to the liver, reabsorbed CBA and UBA are released at the basolateral side of the enterocytes into the portal vein. Finally, they are taken up into the hepatocytes

through the action of *organic anion-transporting proteins* (OATPs) or *sodium-dependent sodium-taurocholate cotransporting polypeptide* (NTCP).

Besides their function in lipid uptake, BA also features signaling function as already indicated. For example, BA induces the activation of G protein-coupled receptor TGR5 (G protein-coupled bile acid receptor 1 also known as GRBAR1) (Thomas et al., 2009). The induced signaling pathways cause organ- and cell type-specific responses. In neuroendocrine L-cells of the intestine, *glucagon-like peptide 1* (GLP-1) is secreted, which in turn contributes to the complex insulin-mediated metabolic regulation (Campbell and Drucker, 2013). In brown adipose tissue as well as muscle, thyroid hormone production is stimulated resulting in enhanced energy expenditure. Furthermore, TGR5 activation is connected with modulation of immune cell responses (Kawamata et al., 2003), and it preserves hepatocellular function including the maintenance of lipid homeostasis (Vassileva et al., 2006; Keitel et al., 2007).

The composition and the levels of BA are also of clinical and pathophysiological importance. Harmful effects of altered BA levels and probably BA composition have been described in different disease patterns. In patients with primary sclerosing cholangitis (PSC) or nonalcoholic fatty liver disease (NAFLD), hepatotoxic effects as a result of a disturbed BA metabolism are thought to play a major role in the development and progression of the respective disease. On the other hand, morbidly obese patients undergoing vertical sleeve gastrectomy have increased levels in circulating BA, mediating at least in part the beneficial effects of bariatric surgery. In these patients, the improved glucose tolerance or weight loss seem to be due to FXR-mediated alterations of the gut microbiota (Ryan et al., 2014). Furthermore, for the treatment of diabetes, NAFLD or primary biliary

**Figure 20.2** Structural isomerism of the isobaric bile acids  $\omega$ -muricholic acid ( $\omega$ -MCA), cholic acid (CA),  $\beta$ -muricholic acid ( $\beta$ -MCA), and  $\alpha$ -muricholic acid ( $\alpha$ -MCA).



cirrhosis BA-based therapeutic approaches often make use of synthetic FXR-ligands like norUDCA or  $6\alpha$ -ethyl CDCA (Stanimirov et al., 2012; Mudaliar et al., 2013).

Taken together, BA metabolism is regulated by the concerted action of the nuclear receptors LXR and FXR. Amphiphilic BAs are not only important in dietary lipid absorption but also serve as antimicrobial agents and signaling molecules, and they play a role not only for the development but also for the therapy of certain metabolic diseases. Since the biological function of distinct BAs is dependent on its chemical structure (Figure 20.2), it is of great importance to have access to an appropriate analytical platform to adequately quantify cholesterol and BA species.

## 20.2 Analytical Platform for Bile Acids

The analysis of BA has a long history in clinical research, where targeted BAs are analyzed especially in blood samples for clinical monitoring of patients (Stein et al., 1999). BAs are present in nearly every part of the mammal body, and thus a targeted metabolomics method should cover at least the most relevant matrices, for example, serum, plasma, fat, feces, urine, liver and kidney tissues, and cell cultures. Furthermore, applications have to cover a broad spectrum of BA species, which are particularly structurally similar due to their identical steroid backbone (Bobeldijk et al., 2008). In general chromatographic techniques coupled to mass spectrometry have established themselves for this purpose more or less

singularly (Dettmer et al., 2007). Gas and liquid chromatography methods have been established accordingly, while, especially in later publications, liquid chromatography may be assumed to have a slightly higher performance due to the easy access to different and specialized chromatographic phases (Sarafian et al., 2015).

A basic challenge in chromatographic separation of BAs is that despite highly selective mass spectrometric detectors like tandem mass spectrometers or high resolution mass spectrometers are used, baseline separation for a vast number of isobaric molecular species has to be achieved. This is especially true as many isobaric target molecules show not only identical molecular masses but also identical fragmentation patterns (John et al., 2014).

While the steroid backbone is of a rather nonpolar characteristic, differences between isobaric structures are often only due to structural differences in the mainly polar side groups. Thus, especially for liquid chromatography applications, a chromatographic method not only has to be generally suitable to retain nonpolar molecules, like the often used C-18 phases, but should also have a high chromatographic selectivity based on polar differences. For GC applications it should be greatly focused on sample preparation as a derivatization step has to be included (Eneroth et al., 1966).

The extraction of BAs and sterols in general from biological samples is mainly done by liquid–liquid extraction. In the past, different variations of the well-known *Folch extraction* or *Bligh and Dyer extraction* have been applied with great success (Folch et al., 1957; Bligh and Dyer, 1959). It has been shown that more stable results are gained if liquid–liquid extraction is carried out under

cooling conditions, for example, 5°C (Grundy et al., 1965). The separation of solid particles like tissues can be achieved using standard mechanisms like centrifugation or filtration. Usage of the supernatant after centrifugation for further analysis tends to show a lower degree of analyte losses compared to filtration techniques. A broad spectrum of suitable filters is available, but polytetrafluoroethylene (PTFE) membranes have been favored especially due to their tolerance to chloroform. Also, filter vials have been applied with success in the analysis of sterols and BAs. Another described method for effective extraction of BAs is the usage of hypercritical fluid (Chaudhury and Chaplin, 1999). Although this method seems to be very effective, it has been rarely adopted due to its difficult application and high expenses.

Sample cleanup by solid-phase extraction (SPE) has been established in a broad spectrum of published methods. However, the selection of the right phase in respect to the analytes is of great importance as an SPE cleanup for BA is nearly always connected with analyte losses (Lee et al., 1997). Normal-phase SPE can be used to greatly remove the amount of nonpolar compounds like triacylglycerols and cholesterol esters, which reduces the need for time-consuming rinsing steps in HPLC analysis and adds a complementary sample cleanup to mainly used reversed phase analytical methods. Thus, an SPE cleanup is advisable in applications where BA shall be analyzed in a targeted manner. In many metabolomics-based biomarker researches, however, concentration patterns calculated from BA and corresponding hydroxy sterols are of great importance. It was shown that a selective sample cleanup for both BAs and hydroxyl sterols is not possible in a single SPE run without a high degree of analyte losses or necessity of separated analysis of several elution fractions. In these cases, simple and fast liquid–liquid extraction protocols without further sample cleanup can be an efficient alternative to the time-consuming and laborious SPE approaches although with this approach a greater focus has to be laid on on-column matrix handling (e.g., rinsing steps to prevent quick column deterioration) (John et al., 2014).

For GC analysis, a derivatization step has to be included in the sample preparation. Derivatization is mostly done using well-known silylation techniques (Setchell et al., 1983). Often different variations of methyl-, dimethyl-, *n*-butyl-dimethylsilyl ethers, and other derivatives of BA have been used widely in GC analysis (Suzuki et al., 1997). Early publications also show that hexafluoroisopropyl ester-trifluoracetyl derivates of BA can be used (Imai et al., 1976). To prevent dehydration during sample preparation, a prior derivatization of BA with *o*-methylhydroxylamine to their oximes may be used (Suzuki et al., 1997).

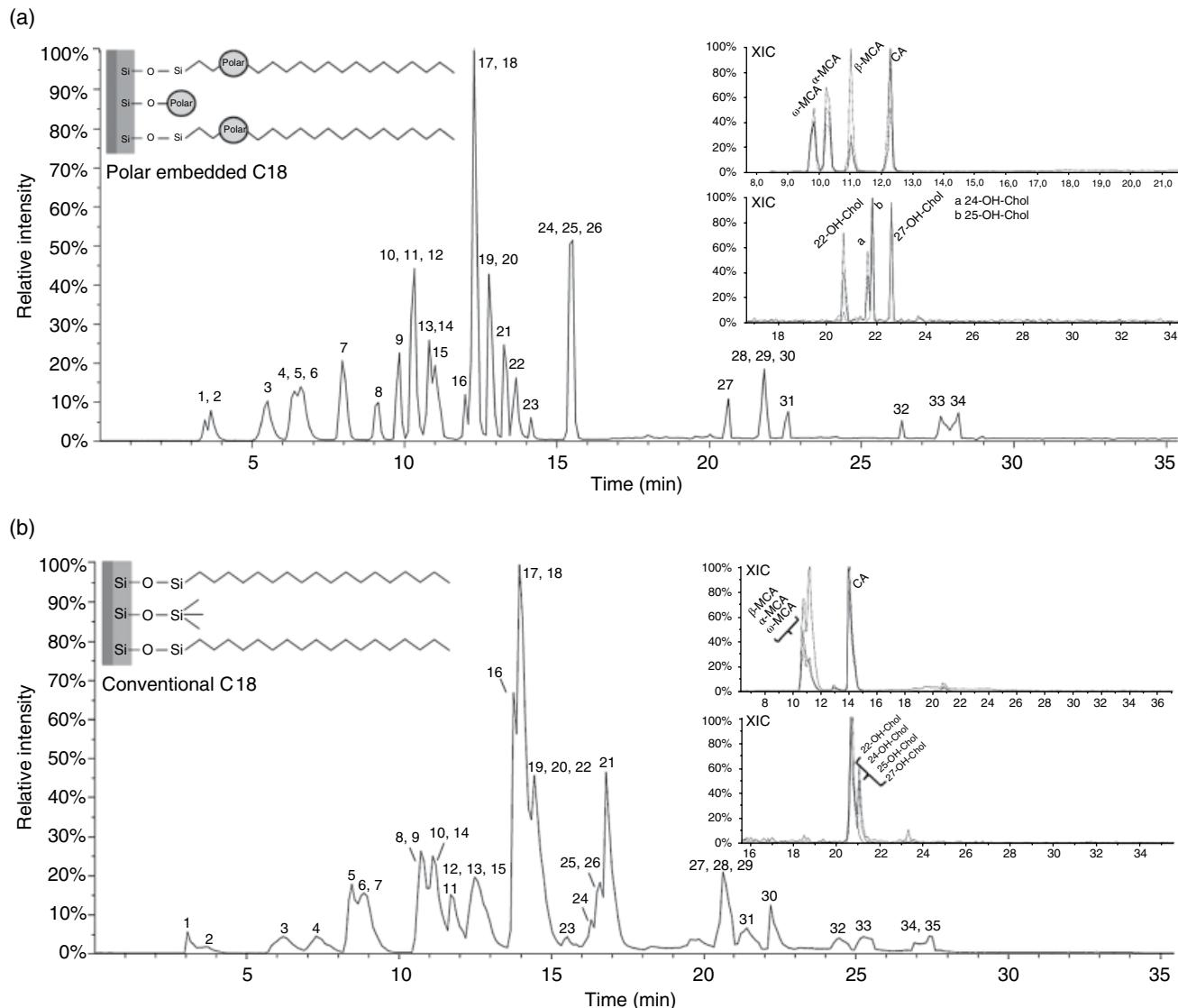
Separation via HPLC has mainly been done by common C-18 reversed phases (Bobeldijk et al., 2008).

This leads to good separation results with common gradients using water, acetonitrile, and isopropanol. Additives used are formic acid, ammonium formate, or ammonium acetate. As often, not all isobaric BAs with similar fragmentation patterns can be baseline separated; the calculation of summed signals is common practice. As an alternative, separation results may be improved using polar-embedded C-18 phases (Figure 20.3) (John et al., 2014). The significant gain of resolution from polar-embedded C-18 phases for BAs and often co-analyzed hydroxyl sterols can be explained by the structural build of BA species. While the steroid backbone, which is to a great extent identical for all species, is of rather nonpolar character, different and characteristic side groups often show polar characteristics. Thus on polar-embedded C-18 phases, general retention is guaranteed based on nonpolar interactions, while selectivity and thus resolution are gained in respect to small polar interactions.

For HPLC-MS applications, ESI is used in most applications though APCI does show good ionization efficiency but tends to complicate structure identification due to in-source fragmentation of the characteristic side groups of BA (Goto et al., 1998; Griffiths, 2003; You et al., 2005). Also MALDI has been adopted to BA analysis in plasma and urine with success, which leads to very short sample preparation and analysis times but limited selectivity especially for isobaric structures (Mims and Hercules, 2003; Mims and Hercules, 2004). For GC-MS applications, electron ionization (EI) and chemical ionization (CI) have been applied depending greatly on the used derivatization technique (Shimada et al., 2001).

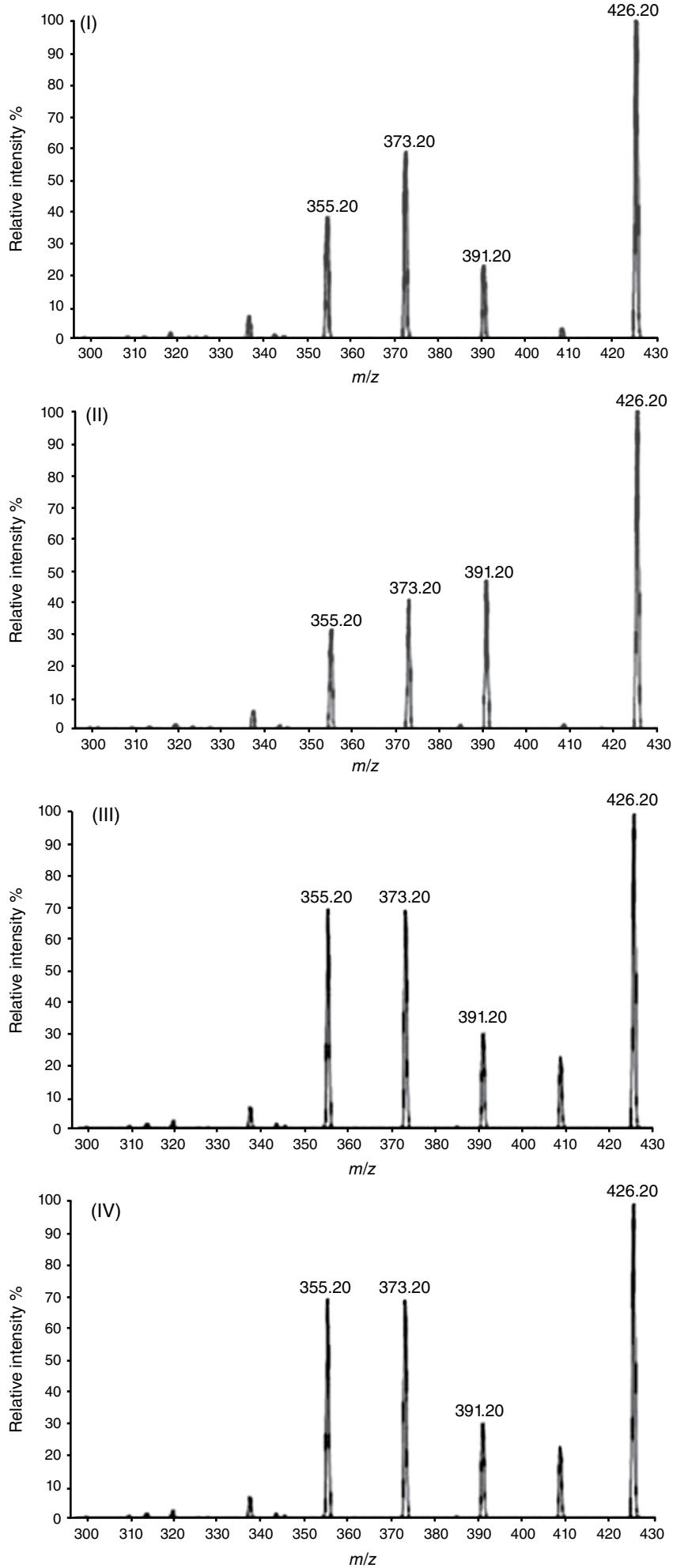
Fragmentation of BA is characterized by fragmentation of hydroxylic and carboxylic side groups at low fragmentation energies and fragmentation of the steroid backbone at higher fragmentation energies (Griffiths, 2003). However, in many cases high dimensional MS applications (e.g., MS<sup>2</sup> or MS<sup>3</sup> experiments) do not lead to higher selectivity due to identical fragmentation patterns as shown for  $\alpha$ -,  $\beta$ -,  $\omega$ -MCA, and cholic acid in Figure 20.4 (John et al., 2014). Therefore, the determination for these analytes is carried out as a sum as baseline separation is not possible (Li-Hawkins et al., 2002).

Using targeted methods, profiling of up to 147 BA species has been applied to perform metabolomic studies. Smaller numbers of BA species are co-analyzed with many nonpolar nontargeted metabolomics applications (Sarafian et al., 2015). The availability of reference standards for identification and quantification of BA species has greatly improved over the last decade, whereas the availability of some derivatives, for example, sulfated BA, is still limited. For these cases, synthesis of these species from available precursors is an alternative (Sarafian et al., 2015).



(1) T- $\alpha$ -MCA (2) T- $\beta$ -MCA (3) TUDCA (4) THDCA (5) TCA (6) DOC (7) GUDCA (8) GCA (9)  $\omega$ -MCA (10)  $\alpha$ -MCA (11) TCDCA  
 (12) Progesterone (13) TDCA (14)  $\beta$ -MCA (15) THDOC (16) UDCA (17) CA (18) GDCA (19) GCDCA (20) Pregnaneone  
 (21) HDCA (22) TLCA (23) Pregnanolone (24) GLCA (25) CDCA (26) DCA (27) 22-OH-Chol (28) 24-OH-Chol (29) 25-OH-Chol  
 (30) 7-OH-Chol (31) 27-OH-Chol (32) Desmosterol (33) Chol (34) Stigmasterol

**Figure 20.3** Total ion chromatograms (TICs) of 34 bile acids as well as oxidized sterol derivatives separated on (a) polar-embedded C18 column (Accucore™ Polar Premium HPLC column (2.6  $\mu$ m, 150 mm  $\times$  2.1 mm i.d.)) or a normal C18 column (b). In contrast to the separation using a (b) usual C18 column, baseline separation of isobaric compounds (e.g., bile acid:  $\omega$ -MCA,  $\alpha$ -MCA,  $\beta$ -MCA, and CA (Figure 20.2); or hydroxysterols: 22-, 24-, 25-, and 27-hydroxycholesterol) is only achieved on a polar-embedded C18 column. Whereas the conventional C18 column contains only aliphatic alkyl chains (scheme (b)), the polar-embedded stationary phase is equipped with several polar groups that are either located within the C18 chain or on remaining free silica groups (scheme (a)). Source: John et al. (2014). Reproduced with permission of Elsevier. Abbreviations: (1) tauro- $\alpha$ -muricholic acid (T- $\alpha$ -MCA), (2) tauro- $\beta$ -muricholic acid (T- $\beta$ -MCA), (3) tauroursodeoxycholic acid (TUDCA), (4) taurohydrodeoxycholic acid (THDCA), (5) taurocholic acid (TCA), (6) deoxycorticosterone, (7) glycourdeoxycholic acid, (8) glycocholic acid, (9)  $\omega$ -muricholic acid ( $\omega$ -MCA), (10)  $\alpha$ -muricholic acid ( $\alpha$ -MCA), (11) Taurochenodeoxycholic acid (TCDCA), (13) taurodeoxycholic acid (TDCA), (14)  $\beta$ -muricholic acid ( $\beta$ -MCA), (15) tetrahydrodeoxycorticosterone (THDOC), (16) ursodeoxycholic acid (UDCA), (17) cholic acid (CA), (18) glycodeoxycholic acid, (19) glycochenodeoxycholic acid (GCDCA), (21) hydeoxycholic acid (HDCA), (22) taurolithocholic acid (TLCA), (24) glycolithocholic acid (GLCA), (25) chenodeoxycholic acid (CDCA), (26) deoxycholic acid (DCA), cholesterol (Chol), (27) 22-hydroxycholesterol (22-OH-Chol), (28) 24-hydroxycholesterol (24-OH-Chol), (29) 25-hydroxycholesterol (25-OH-Chol), (30) 7-hydroxycholesterol (7-OH-Chol), (31) 27-hydroxycholesterol (27-OH-Chol), (32) desmosterol, (33) Chol, (34) stigmasterol.



**Figure 20.4** Electrospray ionization product ion mass spectra of isobaric bile acids (I)  $\alpha$ -MCA, (II)  $\beta$ -MCA, (III)  $\omega$ -MCA, and (IV) CA (see Figure 20.2), showing similar fragment spectra that only differ in their relative intensities. Source: John et al. (2014). Reproduced with permission of Elsevier.

## 20.3 Summary

Using described targeted and nontargeted metabolomics applications, a great number of BA have been postulated as key metabolites or biomarkers for a broad spectrum of metabolic dysfunctions and diseases like hepatotoxicity (Nunes de Paiva and Pereira Bastos de Siqueira, 2005; Ozer et al., 2008), Crohn's disease (Jansson et al., 2009), cancerogenicity (Bernstein et al., 2005; Chen et al., 2011), chronic active liver disease

(Korman et al., 1974), type 2 diabetes mellitus, hypertriglyceridemia, atherosclerosis, and nonalcoholic steatohepatitis (Thomas et al., 2008). The increasing number of analyzable BAs will lead to a fast-growing knowledge about their biofunctional roles especially in disease developments and may result in the application of key BAs as biomarkers for clinical patient monitoring, whereas the further identification of dysregulated BA pathways may be the starting point for drug discoveries and clinical therapies.

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## 21

### Biomarkers for Vitamin Status and Deficiency: LC-MS Based Approach

*Stanley (Weihua) Zhang\* and Jonathan Crowther*

*Ortho Clinical Diagnostics, Material and Process Services, Raritan, NJ, USA*

\* Current address: Merck Manufacturing Division, Global Vaccine & Biologics Commercialization, West Point, PA, USA

#### 21.1 Introduction to Vitamin and Vitamin Deficiency

Vitamins are a group of organic compounds that are vital to bodily functions of human beings. There are 13 commonly recognized vitamins, which are classified by their biological and chemical activities rather than their structures. Each vitamin refers to a group of compounds that show the same or similar biological and chemical activities. Within these vitamins, four of them (vitamins A, D, E, and K) are water soluble and stay in the body for a short period of time (2–4 days). Nine of them (vitamins C, B1, B2, B3, B5, B6, B9, B12, and biotin) are fat soluble and stay in the body much longer (Lieberman and Bruning, 2007). While most of the vitamins needed by the human body are obtained from food or supplement, a small number of them such as vitamin D are produced within the body (Glossmann, 2010).

The biochemical functions of vitamins cover many different areas. For example, the B group vitamin, which contains the largest number of individual vitamins, plays diversified roles in cell metabolism (Le Marchand et al., 2002). Vitamin D is not only essential for the bone health of humans but may also be related to other health conditions and diseases. Vitamin A is not only best known for maintaining vision health but is also important for growth, development, and maintenance of the immune system (Tanumihardjo, 2011). Vitamin K plays a role in blood coagulation (Davie et al., 1991) and bone metabolism (Vermeer et al., 1995), whereas vitamin E and some vitamin C serve as antioxidants (Herrera and Barbas, 2001; Padayatty et al., 2003).

Deficiency of vitamins can cause serious medical conditions. Vitamin A deficiency may cause nyctalopia or night blindness (Genead et al., 2009). It is also a primary cause of xerophthalmia, which is manifested as corneal

abnormalities, or softening of the cornea (keratomalacia) and ulceration, which may lead to irreversible blindness (Sommer, 1998). Vitamin D deficiency is becoming a worldwide problem and it can cause osteopenia and osteoporosis, osteomalacia, muscle weakness, and increased risk of fracture (Holick, 2007). Deficiency of vitamin K has long been known to cause hypoprothrombinemia (Davidson and Tagnon, 1948). Deficiencies of any of the B vitamins can also lead to health problems. For example, vitamin B12 deficiency is a common cause of macrocytic anemia and has been implicated in a spectrum of neuropsychiatric disorders (Oh and Brown, 2003), whereas insufficient amounts of B6 may result in anemia (Ronnenberg et al., 2000), skin disorders (Mooney et al., 2009), susceptibility to infections (Rall and Meydani, 1993), and depression (Hvas et al., 2004). Although vitamin C deficiency is generally considered to be a disease of historical significance (Hampl et al., 2001) and nowadays only occurs in developing countries due to lack of nutrition, studies have shown that high vitamin C intake may reduce the risk of oral, esophageal, stomach, and breast cancers owing to its antioxidant activities (Fairfield and Fletcher, 2002).

Even though there may be multiple biomarkers for each vitamin deficiency, the best biomarker, in many cases is the direct measurement of the vitamin. When a vitamin is evaluated as a biomarker for its status and deficiency, the analytical considerations often are as follows: (i) which form(s) of the vitamin within its production and metabolism pathways (precursors, metabolic products, etc.) are most representative of its status, (ii) whether these form(s) are suitable for a particular test, and (iii) where the vitamin should be tested (bodily fluids such as blood and urine, tissues, or organs). In this chapter, we discuss several vitamins that are deemed essential to nutrition and health and can be detected by liquid

chromatography–tandem mass spectrometry (LC-MS/MS)-based methodology. We will focus on vitamin D, not only because its deficiency is a worldwide problem but also because there are multiple competing quantification methodologies besides LC-MS/MS. The diversity in assay methods as well as high variability in measurements has caused controversy and confusion in clinical testing. Other vitamin biomarkers, including vitamin A, vitamin C, and vitamin B9, will also be briefly discussed. Vitamins other than those mentioned previously have not been measured by LC-MS/MS-based methods in general; therefore, they are out of the scope of this chapter and are not included.

## 21.2 Detection of Vitamin D by LC-MS/MS and Comparison with Other Methods

### 21.2.1 Vitamin D and Vitamin D Deficiency

Vitamin D maybe the most discussed vitamin in recent years due to its implications in a variety of health conditions and its widespread deficiency throughout the global population (Holick, 2007). It is well established that vitamin D is essential for the bone health of humans. Vitamin D promotes calcium absorption and helps in maintaining adequate serum calcium level to enable normal mineralization of bone. It is also needed for bone growth and bone remodeling by osteoblasts and osteoclasts (Cranney et al., 2007). Clinical trials have shown that oral vitamin D supplements helped prevent fractures (Bischoff-Ferrari et al., 2009). Furthermore, vitamin D deficiency has been implicated in various major diseases in recent years, including cancers (Buttiglione et al., 2011), cardiovascular diseases (McGreevy and Williams, 2011), and autoimmune diseases (Ascherio et al., 2010).

Vitamin D deficiency is increasing in the general population to pandemic level and posing risk to global public health. Based on the Institute of Medicine (IOM)'s dietary reference intakes for calcium and vitamin D, the US Centers for Disease Control and Prevention (CDC) reported that 24% of the US population was at risk of inadequacy and 8% was at risk of deficiency between 2001 and 2006 (Institute of Medicine, 2010). Many opinion leaders in the field have suggested that higher concentration levels should be used to define inadequacy and deficiency, thus placing an even higher percentage of the population at the risk levels (Heaney and Holick, 2011).

### 21.2.2 Target the Right Metabolites

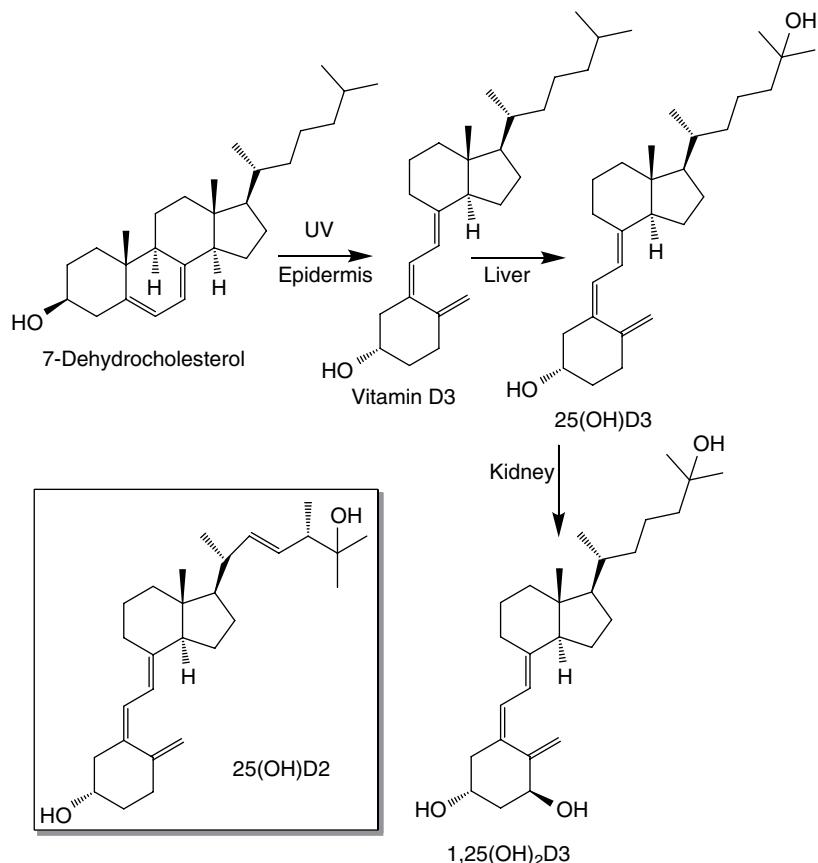
Vitamin D exists in two forms in humans: vitamin D<sub>2</sub> and vitamin D<sub>3</sub>. Vitamin D<sub>3</sub>, the major form of the two, may either be synthesized in skin or obtained from diet.

The human body does not produce vitamin D<sub>2</sub>; therefore the only source is dietary intake. Normally vitamin D<sub>2</sub> levels in the blood are very low and hardly detectable, unless a patient takes vitamin D supplement, which traditionally consists exclusively of vitamin D<sub>2</sub>. Vitamin D is biologically inactive until it is enzymatically converted to active metabolites. Figure 21.1 shows the synthesis and metabolism of vitamin D. Vitamin D<sub>3</sub> is produced from its precursor 7-dehydrocholesterol when skin is exposed to ultraviolet (UV) rays from sunlight. Vitamin D<sub>2</sub> is directly obtained from diet, as mentioned previously. Through enzymatic reactions, vitamin D (including both D<sub>2</sub> and D<sub>3</sub>) is first converted in the liver into 25-hydroxyvitamin D (25(OH)D), the major circulating form of vitamin D, and then in the kidney into 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D), the active form of vitamin D (Christakos et al., 2010). Although 1,25(OH)<sub>2</sub>D is the active metabolite of vitamin D, its serum level does not reflect the body's storage and is not useful for determining vitamin D status (Zerwekh, 2008). 1,25(OH)<sub>2</sub>D also posts analytical challenges due to its low circular concentration (pM vs. nM range) and short half-life (hours vs. several weeks) compared to 25(OH)D (Lips, 2007). Therefore, 25(OH)D concentration is instead universally accepted as a reliable clinical indicator of vitamin D status (Seamans and Cashman, 2009). Most tests in clinical setting report total 25(OH)D, including 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>. However, since the majority of vitamin D supplements consists of 25(OH)D<sub>2</sub>, and there are reports suggesting that 25(OH)D<sub>3</sub> is more potent than 25(OH)D<sub>2</sub> in raising and maintaining serum 25(OH)D concentrations and producing storage of vitamin D (Heaney et al., 2011; Alshahrani and Aljohani, 2013), it is advantageous to have the ability of measuring 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> separately.

### 21.2.3 Analytical Challenges

There are several analytical challenges associated with 25(OH)D quantification. These compounds are very hydrophobic and tend to adsorb to tubes and pipette tips. Thus they are very difficult to handle, especially at low concentration. The hydrophobicity of 25(OH)D also results in coextraction with endogenous lipids from plasma and serum samples in many sample preparation methods, causing issues in chromatographic separation and mass spectrometric detection in high performance liquid chromatography (HPLC) and LC-MS/MS-based methods. Furthermore, 25(OH)D binds strongly to vitamin D binding protein (VDBP) in circulation (Teegarden et al., 1991). VDBP is an approximately 58 kDa glycosylated serum protein that reversibly binds with vitamin D and its metabolites and transports them to target tissues. The affinity constant of the binding

**Figure 21.1** Structures and metabolism of vitamin D.



reaches  $7 \times 10^8 \text{ M}^{-1}$  (Bikle et al., 1986), which compares to that of some antibody-antigen interactions. Therefore, it is important to understand whether the measured 25(OH)D is bound or free when an assay is developed. In addition, the existence of multiple highly resembling 25(OH)D structure analogs requires high specificity for any type of assay. For immunoassays, generation of specific antibodies that can differentiate 25(OH)D from other analogs is extremely challenging and cross-reactivity among antibodies is almost inevitable. On the other hand, one 25(OH)D3 analog, 3-epi-25(OH)D3, has particularly caused issues in HPLC and LC-MS/MS assays, since it poses challenging for both chromatographic separation due to its near identical structure to that of 25(OH)D3 and mass spectrum resolution because of their identical molecular mass. It has long been believed that 3-epi-25(OH)D3 only exists in infants (Singh et al., 2006) and therefore, it has not been evaluated in many quantification assays. However, reports from recent years have provided evidence that 3-epi-25(OH)D3 also presents in adult human beings (Lensmeyer et al., 2012; Engelman et al., 2014), raising the need for a reliable chromatographic separation of 3-epi-25(OH)D3 from 25(OH)D3.

Perhaps the single biggest common issue for the development of a 25(OH)D quantification assay for clinical samples is to obtain reliable reference standards. Since nearly all human blood samples contain some levels of 25(OH)D, there is no true blank matrix available for making calibrator, quality control (QC), or reference samples. Therefore, it remains a challenge to know if the measured concentration is the true value, and the quantification results from different laboratories are often not comparable.

#### 21.2.4 History of Vitamin D Quantification Assays

The earliest evaluation of vitamin D levels was done by various bioassays, most notably, the Rat Line Test, which was first described by McCollum et al. in 1922. Briefly, recently weaned rachitic rats were fed a rachitogenic diet and developed severe rickets. The rats were then fed either diets supplemented with vitamin D3 standards or the unknown test samples. When the radii and ulnae of the sacrificed animals were stained with a silver nitrate solution, silver was deposited in areas of bone where calcium has been recently deposited, and these regions

would turn dark upon exposure to light. Thus the effects of vitamin D<sub>3</sub> in the unknown samples can be visually compared with those of the standards (McCollum et al., 1922). Rat Line Test was not quantitative and therefore could not provide a reading of vitamin D concentration.

Competitive protein binding assays were developed in early 1970s (Haddad and Chyu, 1971; Bayard et al., 1972). In these assays a sample was first subjected to solvent extraction and chromatographic purification to remove its VDBP. The extract (containing unknown quantity of unlabeled 25(OH)D<sub>3</sub>) was then incubated with a known concentration of radiolabeled 25(OH)D<sub>3</sub>, and they competed for an added limited quantity of binding protein. One of the major issues for binding assays is the cross-reactivity with several 25(OH)D<sub>3</sub> metabolites (Dorantes et al., 1978). The assay procedure is very tedious and the performance of automated assay was not acceptable (Glendenning et al., 2003).

Antibody-based assays first emerged in the 1980s as radioimmunoassays (RIAs), which utilized <sup>3</sup>H- or later <sup>125</sup>I-labeled 25(OH)D tracers (Hollis and Napoli, 1985; Hollis et al., 1993). In these assays 25(OH)D was first extracted from human serum or plasma samples by solvent extraction using acetonitrile. Radiolabeled tracer and antiserum against 25(OH)D was then added to the extraction so that the tracer and unlabeled 25(OH)D would compete for the antibody. A second antibody was added to precipitate the 25(OH)D–antibody complex. The pellet was redissolved and radioactivity was counted.

To avoid radiolabeling, enzyme immunoassay (EIA) and chemiluminescent immunoassay (CLIA) were developed. Solvent extraction steps have also been replaced by various denaturing agents that displace 25(OH)D from VDBP and these simplified experimental procedures facilitated automation. In a typical competitive EIA assay, serum and plasma samples were treated with sodium hydroxide to release 25(OH)D from VDBP. These samples were then spiked with biotin-labeled 25(OH)D and incubated with an anti-25(OH)D antibody coated on the EIA plate. Horseradish peroxidase (HRP)-labeled avidin, which binds specifically to biotin that conjugated to 25(OH)D, was added. Color developed when tetramethylbenzoate, a chromogen substrate of HRP, was added, and the absorbance reading is indirectly proportional to 25(OH)D concentration in samples (Hypponen et al., 2007). CLIA assays were based on the same principle as that of the EIA assays. However, instead of using a chromogen substrate in the case of EIA, a chemiluminescent substrate was used in CLIA, which provided much higher sensitivity and better reproducibility and required less reagents than EIA assays (Ersfeld et al., 2004). One major issue of immunoassays for 25(OH)D quantification is the cross-reactivity with other

analogs, such as 24,25(OH)<sub>2</sub>D, which increases in concentration upon exposure to sunlight (Cashman et al., 2015). Sample preparation procedure in some immunoassays may not displace 25(OH)D from VDBP completely; therefore, variation in measurements has been observed (Heijboer et al., 2012). Most immunoassays are designed to measure total 25(OH)D with antibodies cross-reacting with both 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>. However, 25(OH)D<sub>2</sub> was underrepresented in some immunoassays, and this problem caused issues in test subjects who took 25(OH)D<sub>2</sub> as a supplement (Glendenning et al., 2006).

There are two direct detection methods that do not require antibodies or binding proteins: HPLC-UV and LC-MS/MS. The HPLC-UV methods were developed in late 1970s (Eisman et al., 1977; Gilbertson and Stryd, 1977). The sample preparation in earlier methods was very tedious, including multiple steps such as solvent extraction and chromatographic separation. The extracted samples were then analyzed by HPLC equipped with UV detection. Improvements have been made on almost all stages of the HPLC methods since then and current methods have provided reasonable performance and throughput (Lensmeyer et al., 2006).

The development of the LC-MS/MS based methods represented a major advancement in 25(OH)D measurement. LC-MS/MS methods offer superior specificity over HPLC-UV methods and immunoassays, have the advantage of simultaneous quantification of 25OHD<sub>2</sub> and 25OHD<sub>3</sub>, and therefore are currently considered the gold standard for 25(OH)D measurement (Vogeser et al., 2004; Maunsell et al., 2005; Tsugawa et al., 2005). LC-MS/MS quantification of 25(OH)D is the major focus of this chapter, and the workflow is discussed in detail in the following text. One frequent criticism over LC-MS/MS-based methods is that these methods require highly trained operators to perform, and methodology differs between laboratories in terms of sample preparation, HPLC running conditions, and MS detection. Therefore, method comparison and standardization between LC-MS/MS-based methods and other assays will also be reviewed.

## 21.2.5 Quantification of 25(OH)D by LC-MS/MS

### 21.2.5.1 Considerations in Assay Development and Validation

As mentioned earlier, almost all human blood samples contain certain levels of 25(OH)D, so a convenient true blank matrix is not available. This can pose a problem for making calibrator and QC samples. The calibrators may be made in 25(OH)D stripped human serum (Thibeault et al., 2012). Since internal standards are commonly used in LC-MS/MS assays, calibrators can be made in surrogate

matrices such as bovine serum albumin solution (Adamec et al., 2011) or even in a neat solvent (Saenger et al., 2006; Herrmann et al., 2010), since typically internal standard should correct any variation caused by different matrices.

QC samples may be prepared in a surrogate matrix, too, but it is important to evaluate a method using QC samples prepared in an authentic matrix at least at one concentration level to demonstrate the absence of potential bias introduced by using surrogate matrix (Jian et al., 2012). In our laboratory, the low-level QC samples were prepared from a horse serum pool, which contained marginal levels of vitamin D and was screened for its baseline concentration prior to being spiked with a known amount of analytes to reach the designed concentration. Mid-level and high-level QC samples were prepared from a human serum pool, which was spiked on top of the measured endogenous levels to reach the designed concentrations (Zhang et al., 2014).

The endogenous 25(OH)D in human plasma and serum samples should also be considered when the recovery and matrix effects are assessed. In some cases, analyte-free matrices such as charcoal-stripped plasma, where 25(OH)D has largely been removed, have been used (Bogusz et al., 2011). In other cases, the concentrations of endogenous 25(OH)D have been prequantified and incorporated in the calculation of the recovery and matrix effects (Zhang et al., 2014).

The use of internal standards is crucial when dealing with different matrices. Internal standards are normally added to the samples before any sample preparation procedure or, in the case of protein precipitation, to the solvents that used for precipitating protein. In order to track the recovery of analytes more accurately, it is preferred that the internal standards are added to the samples first, and then sufficient equilibration time is allowed for the binding of these internal standards to the VDBP. The most frequently used internal standards are stable isotope labeled 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> (Vogeser et al., 2004; Herrmann et al., 2010), even though some 25(OH)D analogs had been used in early assays (Higashi et al., 2001; Saenger et al., 2006).

Because of the binding of 25(OH)D to VDBP, a 25(OH)D releasing step is almost inevitable in sample preparation procedures. The details of various sample preparation schemes are discussed in the following section.

#### 21.2.5.2 Sample Preparation

The common sample preparation methods for extraction of 25(OH) vitamin D from human plasma or serum samples include liquid-liquid extraction (LLE), solid-phase extraction (SPE), protein precipitation, or various combinations of these methods, depending on mass spectrometer models used as well as other laboratory conditions. The sample volume is normally between 100

and 200 µL, and internal standards are normally added at the very beginning of sample preparation.

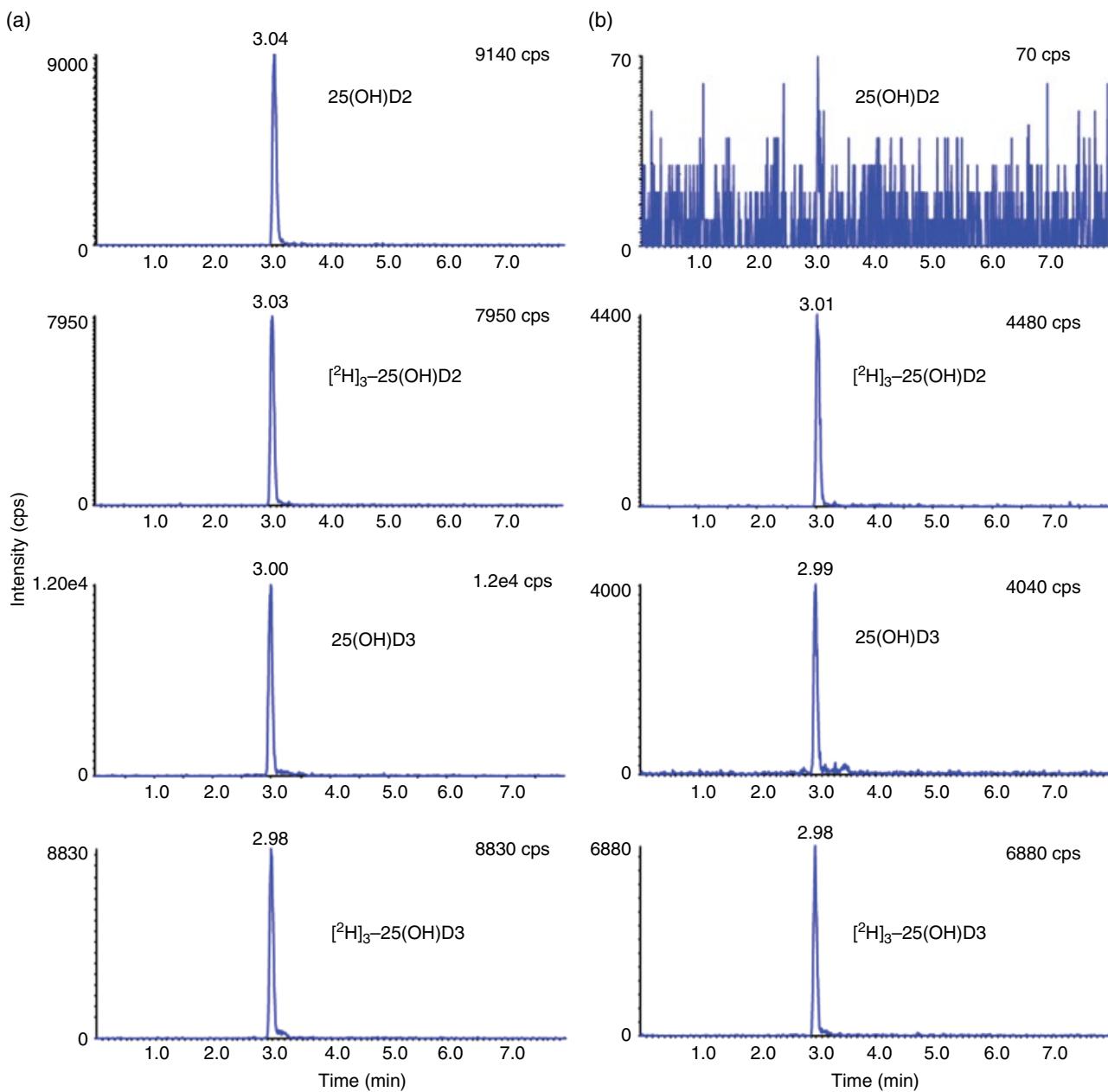
LLE usually yields a relatively clean extraction and it had been used in some early LC-MS methods when triple quadrupole mass spectrometers at that time were not sophisticated enough to handle dirty samples (Maunsell et al., 2005; Saenger et al., 2006). Common solvents include *n*-heptane and hexane. Since 25(OH)D binds to VDBP, often a protein precipitation step is performed to release 25(OH)D prior to LLE (Saenger et al., 2006; Zhang et al., 2014). LLE alone as extraction method has also been reported, and presumably 25(OH)D was released from binding proteins through high pH, mechanical shaking, or sonication (Tai et al., 2010; Adamec et al., 2011; Strathmann et al., 2012). LLE is very labor intensive and automation, though not very straightforward, is necessary to achieve high throughput (Hojskov et al., 2010).

SPE is another sample preparation method for providing clean extraction of 25(OH)D. The most commonly used solid phase material is C18 (Tsugawa et al., 2005). Just as in the case of LLE, protein precipitation is necessary for releasing 25(OH)D prior to SPE procedure (Chen et al., 2008; Knox et al., 2009). Sodium hydroxide has also been reported for 25(OH)D releasing prior to SPE (Vogeser et al., 2004). The cost of performing SPE is high compared to that of LLE. However, SPE is relatively easy to automate, either offline (Knox et al., 2009) or online to LC-MS (Vogeser et al., 2004; Chen et al., 2008).

Protein precipitation, when used alone, tends to yield dirty extraction, thus incur severe matrix effects during detection. With the continuous development of more sophisticated triple quadrupole spectrometers in recent years, matrix effects can be greatly reduced or tolerated and protein precipitation alone without LLE or SPE has become a viable option for 25(OH)D extraction (Herrmann et al., 2010; Bogusz et al., 2011). The most commonly used solvent for protein precipitation is either methanol or acetonitrile. The protein precipitation procedure is very simple and straightforward, and it avoids the phase separation and extraction evaporation of LLE, or the multiple steps required in SPE. Protein precipitation is very easy to automate by using a 96-well filter plate which provides excellent throughput.

#### 21.2.5.3 LC-MS/MS

The HPLC separation of 25(OH)D is mostly achieved by using reverse phase chromatography, including C18 (van den Ouwehand et al., 2010; Bogusz et al., 2011) and C8 columns (Herrmann et al., 2010; Adamec et al., 2011). However, several types of specialty columns are utilized to separate 3-epi-25(OH)D<sub>3</sub> from 25(OH)D<sub>3</sub> (Singh et al., 2006; Strathmann et al., 2012). Methanol is the universal mobile phase solvent used in almost all



**Figure 21.2** Typical mass chromatograms of an extracted 50 ng/mL QC sample (a) and a normal human serum sample (b). MRM transitions: 25(OH)D2,  $m/z$  413.3 → 355.2; [<sup>2</sup>H]<sub>3</sub>-25(OH)D2,  $m/z$  416.3 → 358.2; 25(OH)D3,  $m/z$  401.3 → 365.2; and [<sup>2</sup>H]<sub>3</sub>-25(OH)D3,  $m/z$  404.3 → 368.2. Source: Zhang et al. (2014). Reproduced with permission of Elsevier.

published LC-MS/MS methods. The MS/MS analysis was exclusively performed by triple-quadrupole mass spectrometers. In terms of ionization mode, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) all have been applied (van den Ouwehand et al., 2010; Adamec et al., 2011; Bogusz et al., 2011). It has been suggested that the performance of these ionization modes is somewhat dependent upon specific models of mass spectrometers supplied by different manufacturers

(Bogusz et al., 2011). Figure 21.2 shows representative mass chromatograms of an extracted QC sample which has 25(OH)D2 and 25(OH)D3 each at 50 ng/mL (a) and a normal human serum sample (b).

#### 21.2.5.4 Method Comparison and Standardization

The concentration of 25(OH)D in human blood quantified by different testing methods can vary dramatically. As discussed, since almost all human blood samples

contain some level of 25(OH)D and there is no convenient blank matrix for making QC or reference samples, it is very challenging to know if a measurement is the true value. According to a January 2012 report published by

**Table 21.1** 25(OH)D participants in DEQAS January 2012.

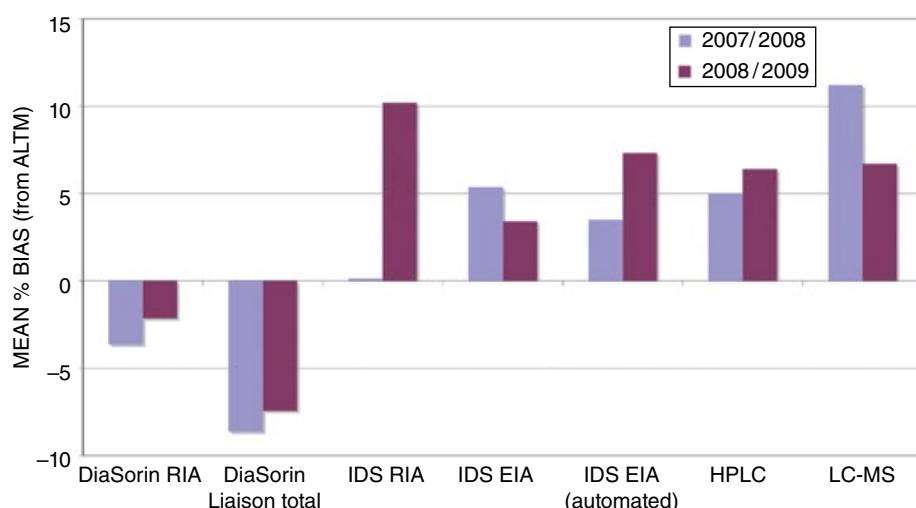
Method	Number of testing by each method	CV for samples analyzed (%)
Diasorin Liaison total	401	9.6–13.7
IDS EIA	136	10.2–11.8
IDS-iSYS	131	8.1–12.9
LC-MS/MS	125	8.9–14.9
Automated IDS EIA	106	8.6–10.2
Roche total 25OHD	61	8.2–16.9
Siemens ADVIA Centaur	46	14.0–19.6
Abbott Architect	34	6.8–10.7
HPLC	31	15.4–35.6
Diasorin RIA	26	14.1–20.7
IDS RIA	11	5.2–15.3
Unknown	4	9.5–24.3
Roche 25OHD3	3	7.1–39.7
DIAsource	2	7.6–36.7
Diazyme 25OHVitD	1	NA
Chromatographic ligand	1	NA

Source: Fraser and Milan (2013). Reproduced with permission of Springer.

NA, not available.

the Vitamin D External Quality Assessment Scheme (DEQAS, refer to more details later in the chapter), the dominant 25(OH)D testing methods were still immunoassays, which accounted for 86% of all the testing covered by the program; the second largest group was LC-MS/MS, which accounted for 11% of all testing. Table 21.1 listed all methods that were utilized by the laboratories who participated in the program, the number of testing that had been done using each method, and the CV for samples analyzed. The Diasorin Liaison 25(OH) Vitamin D TOTAL Assay was the most established immunoassay method and it accounted for about 42% of all immunoassays (Fraser and Milan, 2013). Therefore, the comparison between the Liaison Total assay and LC-MS/MS methods is of high importance and it has been a contentious topic in the field of vitamin D clinical testing.

A 2009 DEQAS report indicated that the measurements by LC-MS/MS were in average about 13% higher than those by the Liaison Total (Figure 21.3) (Vitamin D External Quality Assessment Scheme, 2009). Other research supported this observation. In one study, six other methods were compared with LC-MS/MS, and all immunoassays including Liaison Total showed pronounced underestimation of 25(OH)D3 (Roth et al., 2008). Similar testing from our laboratory showed that LC-MS/MS readings were in average 9% higher than those of Liaison Total (Zhang et al., 2014). A more recent DEQAS report showed that LC-MS/MS methods were more reproducible over longer term compared to the Liaison Total and other immunoassays (Carter et al., 2015). Since in LC-MS/MS methods 25(OH)D2 and 25(OH)D3 are tracked by stable-isotope-labeled internal standards



**Figure 21.3** Relative performance of 25(OH)D methods in the two distribution cycles during 2007–2009. ALTM, all-laboratory trimmed mean; EIA, enzyme immunoassay; Liaison® is a trademark of DiaSorin Inc. (Stillwater, Minnesota, USA); IDS, immunodiagnostic systems holdings PLC (Tyne & Wear, UK); RIA, radioimmunoassay. Source: Carter et al. (2010). Reproduced with permission of Elsevier.

having identical physicochemical behaviors, it is reasonable to believe that these methods provided the highest attainable analytical accuracy and reproducibility.

One possible reason for low readings of Liaison Total assay compared to those of LC-MS/MS is the potential low recovery of 25(OH)D from sample preparation. LC-MS/MS assays use organic solvent for 25(OH)D extraction, whereas the Liaison Total assay is aqueous and relies on pH change and/or blocking agents to liberate 25(OH)D from VDBP. Incomplete recovery could cause underestimation of the concentration in Liaison Total assay. Data from a study that compared Liaison Total as well as other automated assays with LC-MS/MS seemed to support this explanation: an inverse relationship between VDBP concentrations and deviations from LC-MS/MS results was observed (Heijboer et al., 2012).

In order to ensure accuracy and comparability of different 25(OH)D quantification assays, various attempts have been made to standardize vitamin D clinical testing over the years. The National Institute of Standards and Technology (NIST) of the United States released the Standard Reference Material (SRM) 972 in 2009 as control standards for method accuracy (Phinney et al., 2012). SRM 972 were a set of four samples made of either a normal human serum pool (Level 1), a blend of the human serum and horse serum (Level 2), or a human serum pool enriched with 25(OH)D (Level 3) and 3-epi-25(OH)D<sub>3</sub> (Level 4). It has been reported that SRM 972 are not suitable for some immunoassays due to the spiking of exogenous metabolites and the presence of horse serum (Horst, 2010; Bedner et al., 2013). At present the original stock of SRM 972 has been used up and superseded by 972a.

DEQAS was incorporated in 1989 with a goal to ensure the analytical reliability of vitamin D assays ([www.deqas.org](http://www.deqas.org)). Based on its website, it has 1200 participants in 54 countries as of January 2013. DEQAS distributes five samples of unprocessed human serum to its participating laboratories for testing in each quarter. It then gathers and analyzes the test results, and provides a statistic report for all methods as well as each individual method type, including LC-MS, HPLC-UV, and several major immunoassays. The report contains number of tests, mean concentration, standard deviation, and coefficient of variation (Carter et al., 2010). Since it is an average value of a large number of test results, the method mean provided by DEQAS may be used as a surrogate “true value” when evaluating a 25(OH)D assay. In April 2013 DEQAS became an accuracy-based scheme with 25(OH)D values assigned to all samples by the Reference Measurement Procedure of NIST. Participants can now assess the accuracy of their results by comparing them to an internationally recognized reference method (Carter et al., 2015).

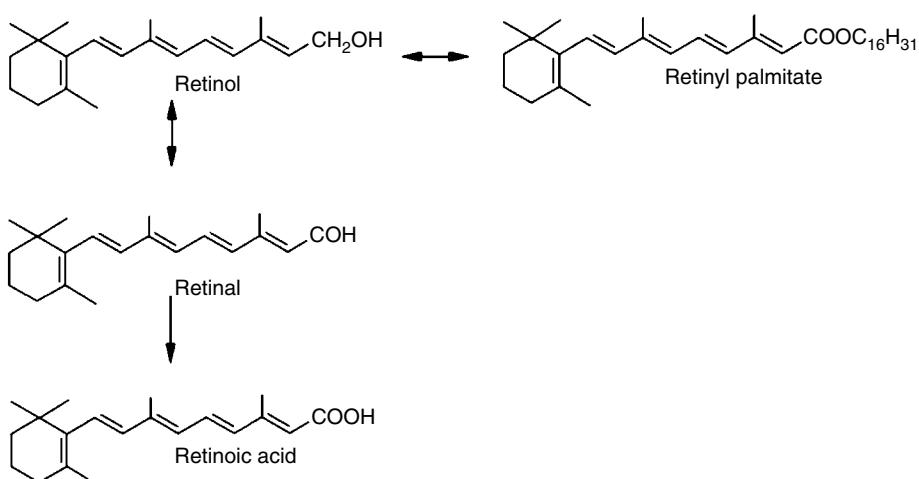
Substantial progress has been made on assay standardization through the Vitamin D Standardization Program (VDSP), which was established in November 2010 by the Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH) in collaboration with the Centers for Disease Control and Prevention (CDC), the National Institute for Standards and Technology (NIST), and Ghent University in Belgium. VDSP is an international collaborative effort to standardize the laboratory measurement of vitamin D status, with a goal of making these measurements accurate and comparable over time, laboratory locations, and assay procedures.

VDSP programs are open to assay manufacturers, clinical and commercial laboratories, national survey laboratories, and research laboratories. The program has a well-defined path for participants to be certified by the CDC Standardization Certification Program. Briefly, the participants need to go through two phases. In phase 1, a participant receives 40 nonpooled, single donor serum samples with reference values assigned. These samples are used to assess the accuracy of a test and to help in recalibrating the test as needed. In phase 2, after the test is calibrated, the participant receives four quarterly challenges of unknown serum samples for analysis. Values reported from participants on these serum samples are used for bias assessment. The performance criteria are 5% mean bias and 10% imprecision. When participants pass four consecutive surveys, they are awarded certification for one year, which is renewable annually. So far VDSP has gained widespread support from clinical testing laboratories, and the major testing providers that have been awarded certificates include LabCorp, Siemens, Immunodiagnostic Systems (IDS), Covance, DiaSorin, Quest Diagnostics, Roche Diagnostics, Abbott Laboratories, and Fujirebio. Standardization of vitamin D assays is essential to patients’ safety and health, and it appears that we are heading in the right direction (CDC Vitamin D Standardization-Certification Program, 2016).

## 21.3 Other Vitamin Biomarkers

### 21.3.1 Retinol: Biomarkers of Vitamin A Status and Deficiency

The most common forms of preformed vitamin A in the human diet are retinol and retinyl esters (Figure 21.4). Vitamin A is essential for various human functions, among which the best known is vision health. The biomarkers of vitamin A deficiency are quite diverse, but the gold standard is the measurement of liver reserves of vitamin A since the liver is the major storage organ. However, this testing is not feasible in human studies.



**Figure 21.4** Chemical structures of important functional forms of vitamin A. Retinol and retinyl esters (palmitate shown) are the dietary forms of preformed vitamin A. Retinal is essential in vision, and retinoic acid is involved in growth and cellular differentiation. Source: Tanumihardjo (2011). Reproduced with permission of American Chemical Society.

Serum retinol concentrations have been used for evaluation of vitamin A deficiency (Tanumihardjo et al., 1994), but the drawback for this method is that serum retinol concentrations are homeostatically controlled until liver reserves are very low (van Lieshout et al., 2003). Currently the most accurate technique to determine vitamin A status in humans is isotope dilution. To perform an isotope dilution experiment, a dose of a labeled tracer, either <sup>2</sup>H- or <sup>13</sup>C-labeled retinyl acetate, is first given orally to a subject. Sufficient time (about 20 days) is then allowed for the labeled tracer to reach equilibration with endogenous body reserves. Blood samples are taken at various times, and concentrations of both labeled and unlabeled retinol are measured by mass spectrometry. The concentration of endogenous reserves is calculated based on the extent of dilution of the labeled tracer (Ribaya-Mercado et al., 1999).

While traditionally GC-MS has been used in isotope dilution experiment, LC-MS/MS has offered superb sensitivity and the potential for high throughput analysis. In a recent study designed for evaluation of vitamin A status and bioavailability/bioconversion of β-carotene, a provitamin A carotenoid, <sup>13</sup>C labeled β-carotene, and retinyl acetate was coadministered orally to human subjects over a 2-week period. An ethanol/ethyl acetate (1:1) solvent mixture was applied to plasma samples to extract all analytes, including β-carotene, retinol, retinyl acetate, retinyl linoleate, retinyl palmitate/retinyl oleate, and retinyl stearate. These analytes as well as any stable isotope labeled counterparts were chromatographically separated using a C18 column in a 7-min run and detected by a triple quadrupole mass spectrometer. Concentrations of both labeled and unlabeled analytes were quantified against external calibration curves made

with unlabeled analytes. Thus coadministration of stable isotope labeled retinyl acetate and β-carotene not only acts as a reference dose for inter-individual variations in absorption and chylomicron clearance rates, but also allows for simultaneous determination of an individual's vitamin A status (Oxley et al., 2014).

### 21.3.2 Folic Acid: Biomarkers for Vitamin B9 Dietary Intake

Vitamin B9, or folates are a group of essential cofactors in several metabolic pathways including biosynthesis of nucleotides and amino acids as well as methylation reactions (Selhub, 2002). Folate has generated great research interests due to its putative role in health and disease (Stover, 2004). Humans cannot synthesize folates, which has to be supplied through the diet to meet our daily requirements. Folates present naturally in many foods and they can also be found in fortified foods or supplements.

LC-MS/MS assays have been developed and validated to quantify various folate forms including tetrahydrofolate (THF), 5-methylTHF, 5-formylTHF, 5,10-methenylTHF, and folic acid in human serum or plasma samples (Hannisdal et al., 2009; Kirsch et al., 2010). Sample preparation methods included SPE and protein precipitation. Folates can also be purified by a folate binding protein-based affinity column (Kok et al., 2004). Chromatography separation was performed mostly on reverse phase columns (C18 and C8), but hydrophilic interaction liquid chromatography (HILIC) columns were also used (Alvarez-Sanchez et al., 2010). In one study, the sum of the folate forms obtained by LC-MS/MS correlated strongly with the concentration values measured by an

immunoassay (Kirsch et al., 2010). Current LC-MS/MS assays present sufficient precision, accuracy, and throughput for quantification of multiple folate forms in large-scale clinical studies.

### **21.3.3 Vitamin C: An Appropriate Biomarker of Vitamin C Intake**

The primary source of dietary vitamin C (ascorbic acid) is fruits and vegetables, whose consumption has been linked to reduced rates of chronic diseases, including cancer, cardiovascular disease, diabetes, and arthritis (Ziegler, 1991; Carr and Frei, 1999; Simon et al., 2001). It is believed that the protective function of fruits and vegetable is mainly attributed to some antioxidant compounds such as vitamin C, which inhibit lipid peroxidation and oxidative cell damage (Steinmetz and Potter, 1991). Although it is not perfect, the vitamin C level in the blood has been considered a reasonable surrogate for dietary vitamin C intake and an index of the circulating vitamin C available to tissues (Dehghan et al., 2007).

HPLC methods with electrochemical detection, which provide necessary sensitivity and specificity, are generally used to quantify serum vitamin C concentrations (McCoy et al., 2005). LC-MS/MS based methodology has also been explored. In a recent publication, Szultka and colleagues described an LC-MS/MS method for quantification of vitamin C and its degradation products in the presence of reactive oxygen species in a water-based solution. The determination of vitamin C and its degradation products was carried out using C18 reverse phase chromatography coupled with a triple quadrupole mass spectrometer. No published data have been found on application of LC-MS based methods for vitamin C testing in human blood samples. However, Quest Diagnostics has patented mass spectrometry detection of vitamin C (Jiang et al., 2011) and the company offers an LC-MS/MS based test in its test menu.

## **21.4 Conclusions and Perspectives**

It has long been established that vitamins have diverse biochemical functions and are crucial to maintain human health. Both government agencies and private, nonprofit institutions have acknowledged the roles of vitamins in healthcare and take actions to promote their dietary intake. For example, U.S. Department of Health and Human Services (HHS) and the U.S. Department of Agriculture (USDA) publish a dietary guideline every five years. These guidelines provide detailed information on suggested daily value of dietary intake of each vitamin, a list of foods that contain these vitamins, as well as how much vitamins these foods contain (Department of

Health and Human Services and Department of Agriculture, 2005). The IOM, a division of the National Academies of Sciences, Engineering, and Medicine, issued reports on dietary reference intakes on vitamin D (Institute of Medicine, 2010), vitamin A, vitamin K (Institute of Medicine, 2001), vitamin C, vitamin E, carotenoids (Institute of Medicine, 2000), and B vitamins (Institute of Medicine, 1998). The missing link, however, is accurate clinical testing. Following suggested daily dietary intake of a vitamin does not necessarily translate into sufficient level of it in human body, and a proper laboratory test is the only sure way to assess the vitamin level. As of now, testing of vitamins is not covered in annual preventive care examination by major health insurance providers. Vitamin D testing has been covered by some insurance policies for several years, but many of these policies have since dropped the coverage.

The main argument for noncoverage is that there is no sufficient scientific and clinical evidence to support the benefits of vitamin screening. As an example, in a recent publication the U.S. Preventive Services Task Force (USPSTF or Task Force) did not recommend screening for vitamin D deficiency in adults because the task force concluded that “the current evidence is insufficient to assess the balance of benefits and harms of screening for vitamin D deficiency in asymptomatic adults” (LeFevre, 2015). The evidences cited were: the current test methods were not sufficiently accurate and consistent; there was lack of consensus on the laboratory values that define vitamin D deficiency; and the detection and treatment of asymptomatic vitamin D deficiency presumably has no benefit on cancer, type 2 diabetes, and other health conditions. While there is truth in these arguments, many of these aspects will change overtime: the accuracy of the test methods has already been improved greatly; efforts on method standardization are underway, therefore greater consistency should be achieved; and there is reasonable consensus on definition of inadequacy and deficiency (Institute of Medicine, 2010). While the link between the vitamin D deficiency and diseases such as cancer and cardiovascular diseases is not conclusive, more clinical trials are underway to elucidate any potential connections (Manson and Buring, 2016). Considering these arguments as well as the firm evidence between vitamin D and bone health, vitamin D tests should be included in routine physical examination.

Another reason that vitamin testing is not reimbursed during routine physical examinations is the cost. Current test methods further exacerbate the issues of cost and complexity since each vitamin is tested individually. Technology of multiplexing may help alleviating financial burden. LC-MS/MS technology is especially well equipped with multiplexing capacity based on its ability of simultaneously analyzing multiple analytes in a single

run. In fact, a method to quantify multiple fat soluble vitamins in human serum has been developed (Priego Capote et al., 2007). Assays for measuring water soluble vitamins have also been developed, albeit not yet applied in clinical samples (Chen et al., 2009; Doyle, 2013). It can be reasonably predicted that the majority of the vitamins can be quantified within two or three LC-MS/MS runs in human samples in the near future. Once these methods are established in clinical setting, the cost of running vitamin testing will be reduced dramatically.

Vitamin profiling (screening vitamin deficiency in general population) and personalized vitamin may present an opportunity to improve the public health. Based on the results of vitamin profiling, an individual can make specific changes in dietary intake and lifestyle to

counter any deficiencies, thus improving health conditions. Right now the personalized vitamin strategy is undertaken mostly by private companies and the situation is chaotic at best. Some companies simply use the terms of "personalized vitamin" and "vitamin profiling" for marketing purpose. In many cases decisions are made based on online questionnaires rather than clinical testing. Healthcare authorities should seize the opportunity to provide regulation to the industry. Specific vitamin decisions of individuals should be made based on proven clinical testing procedures. Ultimately, with proper monitoring and balanced vitamin status, general health outlooks may be improved and chronic diseases prevented, which lead to reduction of overall healthcare cost.

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## 22

### Quantitation of Acyl-Coenzyme A Thioesters as Metabolic Biomarkers

Nathaniel Snyder

A.J. Drexel Autism Institute, Drexel University, Philadelphia, PA, USA

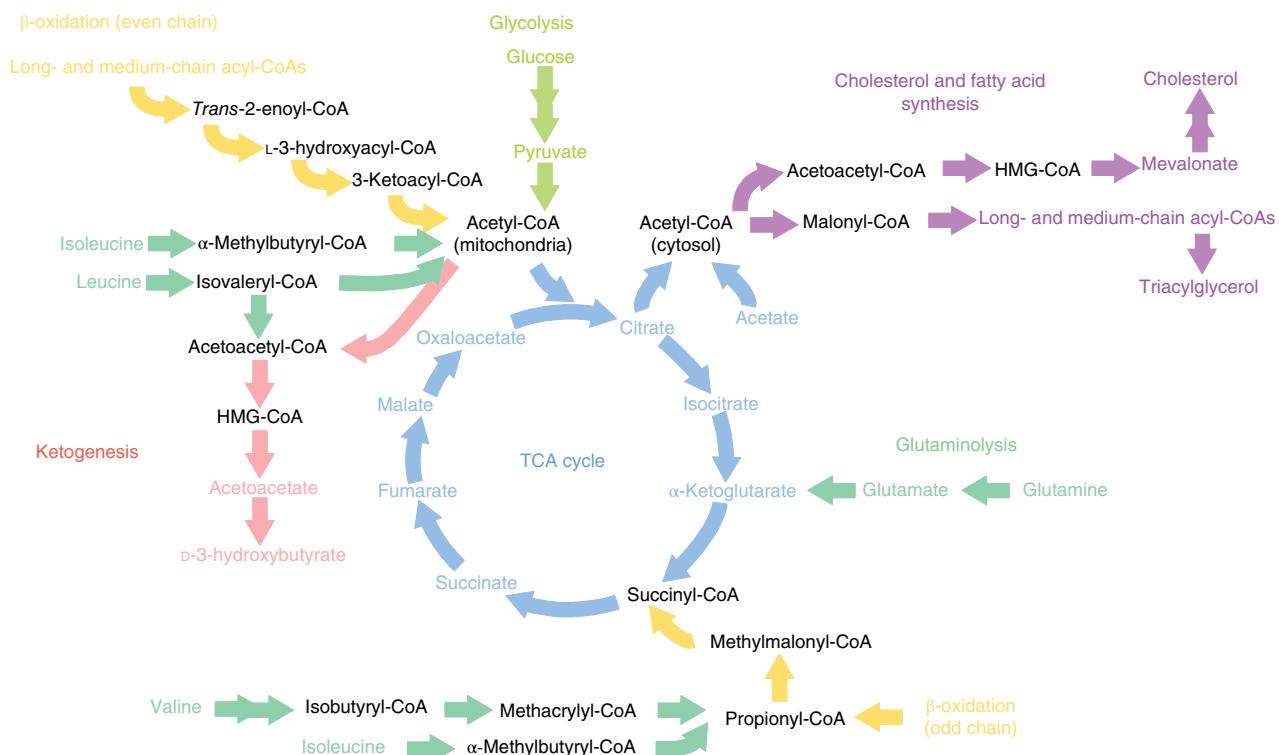
#### 22.1 Introduction

Coenzyme A (CoA) is a ubiquitous, evolutionarily conserved, metabolically critical thiol found in all forms of cellular life. The free sulphydryl group of CoA reacts with carboxyl groups to form thioesters, known as acyl-CoAs. Acyl-CoAs are intermediates in numerous metabolic processes including, but not limited to, lipid metabolism, xenobiotic metabolism, redox maintenance, cell signaling, transcriptional regulation, post-translational modification of proteins, and central carbon metabolism including the tricarboxylic acid (TCA) cycle (Figure 22.1). Thus, bioanalysis of acyl-CoAs is useful not only for understanding biochemistry and pathological basis of disease but also for developing and testing therapeutics for a wide range of diseases. Although the CoA moiety is conserved across metabolites within the class of acyl-CoAs, thioester linkage to a diverse range of potential acyl groups makes acyl-CoA analysis challenging in terms of specificity, sensitivity, generalizability, and robustness. To further complicate bioanalysis of acyl-CoAs, the inherent instability of the thioester linkage, which is not resonance stabilized to the degree of an oxoester, necessitates adjustment by proper quality control and, ideally, use of internal standards that recapitulate the instability of the target acyl-CoAs. Use of LC–MS/MS and, increasingly, LC-HRMS is ideal due to the ability of these platforms to meet the requirements for acyl-CoA analysis. This chapter will discuss the structure and function of acyl-CoAs, provide an overview of the LC–MS-based methods of quantifying acyl-CoAs, and provide specific examples of the analysis of acyl-CoAs as biomarkers for current drug targets, metabolic diseases, and drug metabolism.

#### 22.2 Structure and Function of Acyl-CoAs

The CoA backbone is derived from three components: ATP, a cysteinyl group from cysteine, and a pantethine group, which originates from pantothenate (vitamin B5) (Figure 22.2, top). Plants, fungi, and many prokaryotes are capable of synthesizing pantothenate from its constituents  $\beta$ -alanine and pantothenic acid. However, animals including humans, lack enzymes for de novo pantothenate synthesis and, as a result, require it in their diet. From pantothenate, CoA is synthesized through five enzymatic steps requiring four molecules of ATP. The enzyme that catalyzes the first and rate-determining step of CoA synthesis, pantothenate kinase, converts pantothenate to pantethine-phosphate (Robishaw and Neely, 1985). In addition to regulation via CoA synthesis, the sub-cellular localization of acyl-CoAs in eukaryotic cells is tightly controlled (Grevengoed et al., 2014). Due to the inability of acyl-CoAs to diffuse across cellular membranes, their localization is controlled by a system of acyl-transferases, utilization of carnitine as an acyl-carrier, and tightly regulated carnitine-specific transporters. The mitochondria and cytosol are host to major pools of acyl-CoAs but less well-understood pools of acyl-CoAs also exist within the peroxisome and lysosome. There is some biochemical reasoning to suggest that the cytosolic pool is linked to the nuclear pool but little direct experimental evidence (Zecchin et al., 2015).

Although the free thiol of CoA (CoASH) itself is a major intracellular thiol, it is mostly found conjugated as an acyl-CoA through a thioester linkage to endogenous and exogenous molecules. Unlike the typical ester bond, a thioester is not resonance stabilized, and thioesters are

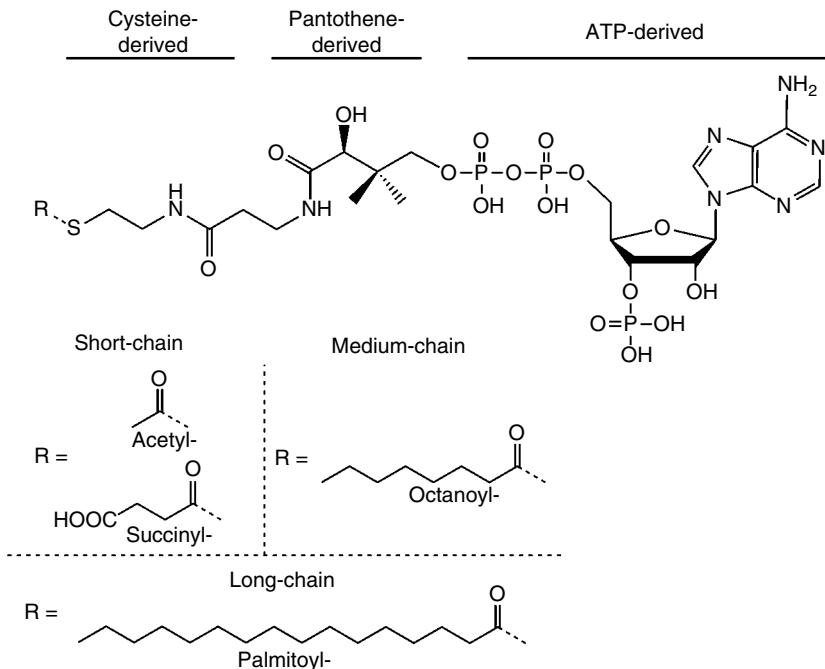


**Figure 22.1** Metabolic map including the involvement of acyl-CoAs in  $\beta$ -oxidation, glycolysis, cholesterol and fatty acid synthesis, glutaminolysis, branched chain amino acid catabolism, ketogenesis, and the Tricarboxylic acid (TCA) cycle, also known as the Krebs cycle.

prone to base catalyzed hydrolysis and to a tran-acylation reaction with nucleophiles (Gil et al., 2015). Reactivity to nucleophiles, which varies by nucleophile in an incompletely understood manner and by structure of the thioester-containing molecule, is anywhere from 100- to 2000-fold more reactive than the equivalent oxoester (Yang and Drueckhamer, 2001). The diversity of acyl-CoA thioesters is wide-ranging, with the potential for unique acyl-CoAs to be derived from each individual fatty acid, and most carboxylic acid-containing metabolites. To better describe and delineate this breadth of possible structures, it is useful to categorize acyl-CoAs based upon the length of their acyl chain as such: short-chain acyl-CoAs (up to 6 carbons), medium-chain acyl-CoAs (6–10 carbons), long-chain acyl-CoAs (up to 20 carbons), and very long-chain acyl-CoAs (greater than 20 carbons) (Figure 22.2, bottom). Short-chain acyl-CoAs comprise, arguably, the most abundant and biochemically important class of acyl-CoAs and are generally water soluble. Longer chain acyl-CoAs are highly variable in their biological abundance and are differentially soluble in water. Acetyl-CoA is a short-chain acyl-CoA of particular import. It is a central metabolic intermediate in two-carbon metabolism and the point by which pyruvate enters the Krebs cycle for oxidation. Succinyl-CoA is another significant acyl-CoA that is also

an intermediate in the Krebs cycle. It plays an important role in facilitating the incorporation of breakdown products of glutaminolysis and branched chain amino acid (BCAA) into the Krebs cycle through anaplerotic reactions. Malonyl-CoA is another important short-chain CoA, which is both a precursor and a regulator of fatty acid catabolism and synthesis. Other functionally significant acyl-CoAs include propionyl-CoA and methylmalonyl-CoA, which are anaplerotic substrates derived from branched-chain amino acids and odd-chain fatty acid metabolism. Acetoacetyl-CoA and  $\beta$ -hydroxybutyryl-CoA, the latter of which exists as two functionally distinct isomers (D/L), are involved in a number of pathways including ketone body and fatty acid oxidation, while 3-hydroxymethyl-3-glutaryl-CoA (HMG-CoA) is a key entry point into ketogenesis and cholesterol synthesis. Medium- and long-chain acyl-CoAs are also diverse in their function and distribution, performing roles as transcription factor binding partners, and predominant roles as intermediates of fatty acid synthesis, polar lipid synthesis, and fatty acid oxidation. Other acyl-CoA species perform a range of biological functions, including a number of intermediate metabolites specific to plant and prokaryotic species, such as cinnamoyl-CoA, an intermediate of phenylpropanoid synthesis found extensively in plants (Barakat et al., 2011).

**Figure 22.2** Structure of CoA (top) and potential acyl-groups (short-chain, medium-chain and long chain). The CoA molecule forms a thioester with a diverse range of acyl-groups.



Other atypical classes of CoA derivatives exist, with biological functions outside of carbon metabolism. One such group, the CoA-disulfides, consists of a number of CoA adducts bound to exogenous or endogenous electrophiles. Similar to glutathione (GSH), the most well-known cellular reducing agent, CoASH can react with xenobiotic compounds and electrophiles. In bacteria that lack GSH, the major reducing intracellular thiol is CoASH. Within systems that produce both CoASH and GSH, the mixed disulfide glutathione-CoA (CoASSG) has been observed but its exact role and mechanism of formation remain unclear. The ratio of CoASSG:CoASH, however, has been shown to increase in settings of mitochondrial oxidative stress such as hepatotoxicity (Rogers et al., 2000). This is just one example of various new roles for CoA which continue to be discovered. Recent discoveries include CoA-linked RNA adducts (Kowtoniuk et al., 2009), CoA-electrophile adducts (Rudolph et al., 2009), and S-nitroso-CoA species (Anand et al., 2014). In fact, the acyl-CoAs described above are but a fraction of the full family of acyl-CoAs. Ongoing studies of human biochemistry have revealed new acyl-CoAs such as the recent discovery of 2-methyl-2-pentenoyl-CoA derived from propionyl-CoA (Snyder et al., 2015a), the functional relevance of which is yet to be determined. And there are, doubtless, many more that remain to be described in both endogenous human biochemistry and xenobiotic metabolism. The numerous established and novel roles for CoA as well as studies tying perturbations in physiological concentrations of acyl-CoA species to pathological settings—including diabetes, heart failure,

liver metabolism, and various inherited metabolic disorders to be discussed below—have spurred efforts to develop methods to better quantify these species.

## 22.3 Detection and Quantitation of Acyl-CoAs

Acyl-CoAs perform versatile and critical biological functions in the cell; they also offer a unique bioanalytical target for metabolic studies which can be exploited in both high-performance liquid chromatography (HPLC) and mass spectrometry (MS) techniques as a basis for development of sensitive and specific assays. Historically, acyl-CoA detection was performed by HPLC-UV with absorbance at 230 and 290 nm, with a maxima reported from 250 to 270 nm. However, this approach is not particularly sensitive, with reported limits in the pmol or nmol range and targeting a moiety conserved across all CoAs severely restricted assay specificity and thus the ability to differentiate between CoA-containing molecules and potentially even other ADP- and thioester-containing compounds. Derivatization and fluorometric detection provided additional sensitivity and can estimate total CoA pools, but was laborious and still limited by the specificity of the derivatization (Demoz et al., 1993). Radiometric detection was possible using a number of different labels, but incurred the constraints of radioactivity on measurement and experimental design (Watmough et al., 1989). The first steps toward using mass spectrometry for CoA measurement were fast

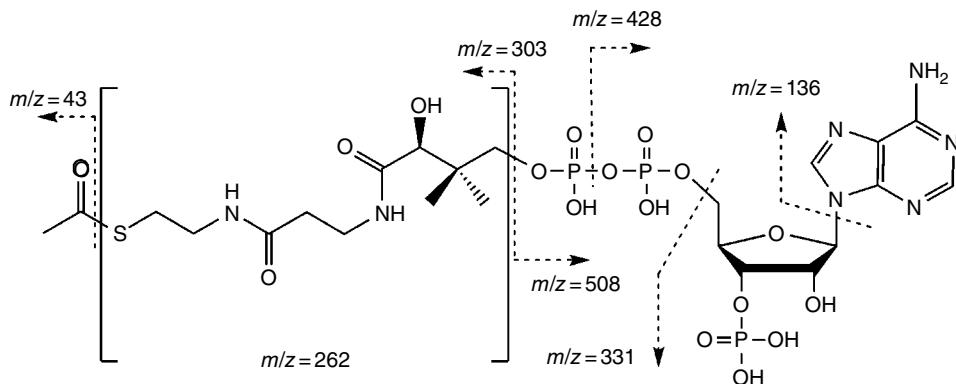
atom bombardment (FAB) MS and derivatization coupled to GC-MS, paving the way for the introduction of LC-MS/MS using electrospray ionization (ESI) reaching fmol levels of detection (reviewed in (Haynes, 2011)).

A necessary first step in acyl-CoAs analysis, owing to their identity as intracellular metabolites in complex biological milieus, is purification via quenching and extraction procedures before introduction onto LC-MS instrumentation. Due to the base-catalyzed hydrolysis of thioesters, and the potential for acyl-transfer, extraction conditions should be tested for variable recovery by pH, temperature, and organic solvent composition. For a number of endogenous acyl-CoAs, an extraction solvent with pH below 6.7 gave good recovery, with stable long-term storage at or below -20°C, and short-term storage in autosampler vials over days at 4°C (Liu et al., 2015). Addition of an internal standard, as discussed below, as early in a bioanalytical process as possible, is advisable based on the above considerations and prior literature (Magnes et al., 2008; Ciccimaro and Blair, 2010). Samples are often quenched with chilled perchloric (Corkey et al., 1981), sulfosalicylic (Demoz et al., 1993), or trichloroacetic acid (Basu et al., 2011), followed by a range of various processing methods ranging from liquid-liquid extractions (LLE) to solid-phase extractions (SPE). LLE using Bligh-Dyer-derived aqueous/methanol/chloroform extractions have been used (Demoz et al., 1993), as have buffered isopropanol/acetonitrile extractions (Minkler et al., 2008). Owing to the diversity of the acyl-groups, themselves impacting the physicochemical properties of various CoAs, most extractions suffer from widely varying recoveries, with incomplete partitioning of acyl-CoAs between the polar and nonpolar phases, with the exception of a more recent SPE-based method using anion-exchange (Minkler et al., 2008). Ultimately, purification and, often, sample concentration before analysis allow de-salting and removal of interfering lipid species, leading to improved sensitivity and robustness of downstream analysis.

LC-MS/MS has seen a sharp rise as a preferred method for bioanalysis of acyl-CoAs (Haynes, 2011). Adaptations from HPLC to LC-MS/MS for the previously reported HPLC conditions included the replacement of phosphate buffers with MS-compatible buffers and reducing the additive concentration. Water, acetonitrile, and/or methanol, usually using ammonium acetate as a solvent modifier, are the most common mobile phases reported for LC-MS/MS analysis of acyl-CoAs. Ion-pairing reagents have been reported in CoA analysis, especially for malonyl-CoA (Gao et al., 2007). C18 and, to a lesser extent, C8 stationary phases have been reported with some use of trapping systems, which introduce additional complexity to the LC system but have logical appeal in covering a wider range of

acyl-CoAs. Most commonly, positive ion mode is used, as acyl-CoAs appear to ionize well in this mode. "Wrong-way" ionization, using negative mode at neutral or slightly acid pH, has been reported and provided excellent analytical figures of merit (Haynes, 2011). Some concerns over using high pH mobile phase with a thioester should be noted, as base catalyzed hydrolysis of the thioester bond is likely and should be monitored. Positive ion formation of acyl-CoAs mostly results in the  $\text{MH}^+$  ion, with some species (the CoA disulfide) producing a doubly charged and protonated  $[\text{M}+2\text{H}]^{2+}$  ion. The fragmentation of the precursor  $\text{MH}^+$  ion has been well described, with high-efficiency fragmentation from a neutral loss of 507 amu ( $\text{MH}^+-507$ ) (Figure 22.3). This results from the fragmentation at the phosphate and loss of ADP (Gao et al., 2007; Frey et al., 2016). Additional high-intensity fragments responsible for the other major peaks in the spectra are derived from the pantethine ejection and the adenosine base. Importantly, the neutral loss as well as the pantethine ejection maintains the acyl-group on the product ion, allowing analysis of the acyl-group. Surprisingly, little work has been published on the higher order fragmentation of acyl-CoAs (e.g.,  $\text{MS}^3$ ).

High-resolution MS (HRMS) offers a particularly attractive avenue for acyl-CoA analysis, led by advances in the robustness, capabilities, ease of use, and reduced cost for HRMS platforms. Time of Flight (ToF), Orbitrap, or ion cyclotron resonance provide high mass resolution, exceeding the nominal mass capabilities of quadrupoles predominantly used for acyl-CoA analysis thus far (Xian et al., 2012). HRMS provides increased multiplexing, specificity, and sensitivity, thus increasing the range of uniquely identifiable and quantifiable metabolites. Utilization of full scan mode in HRMS to provide a wide coverage of an  $m/z$  range allows highly multiplexed and untargeted acyl-CoA analysis, as well as data analysis on secondary analytes not originally quantified but still acquired as part of the experiment (Liu et al., 2015). Hybrid instruments also increase the potential to use alternative scan modes to improve sensitivity or applications. Liu et al. were able to quantitatively distinguish  $\beta$ -hydroxybutyryl-CoA from hydroxyl-isobutyryl-CoA within a highly multiplexed experiment on a quadrupole/Orbitrap hybrid platform by an additional fragment derived from cleavage in the acyl-chain (Liu et al., 2015). At sufficiently high resolution, acyl-CoAs that are nominally isobaric on traditional systems can be resolved through MS alone, reducing the need for more extensive and complex chromatography. As initially described by Gao et al., two major acyl-CoAs—malonyl- $(\text{C}_{24}\text{H}_{38}\text{N}_7\text{O}_{19}\text{P}_3\text{S})$  and (D/L)- $\beta$ -hydroxybutyryl-CoA ( $\text{C}_{25}\text{H}_{42}\text{N}_7\text{O}_{18}\text{P}_3\text{S}$ )—are nominally isobaric ( $m/z$  854.1) (Gao et al., 2007). Furthermore, the predominant single reaction monitoring transition ( $854 \rightarrow 347$ ) as well as



**Figure 22.3** Fragmentation of acetyl-CoA. MS/MS of acetyl-CoA is well described, with a predominant neutral loss of 507 ( $m/z$  810 → 303). Other fragments have been reported for acyl-CoAs, allowing monitoring of confirming ions.

other identified product ions (136, 245, 303, 428, 508, and 543) is identical for these biochemically distinct acyl-CoAs. With 70,000 or more resolving power a 1:1 mixture of these two important species can be baseline resolved at  $m/z$  854.1229 versus 854.1593 (Frey et al., 2016). Also, methods for acyl-CoA analysis on unit resolution platforms suffer from interference from isotopic overlap from highly abundant acyl-CoAs on unsaturated derivatives from the M+2 isotopologue of the more saturated fatty acyl-CoA. This was especially acute in the biochemically important case of the M+2 isotopologue of 18:1 oleoyl-CoA in the 18:0 stearoyl-CoA channel that required resolution by LC (Magnes et al., 2008). With HRMS, less abundant but naturally occurring isotopes ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^2\text{H}$ ,  $^{32}\text{S}$ ,  $^{33}\text{S}$ ,  $^{36}\text{S}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ) and double bonds can be resolved by the MS alone due to mass defects not discernable by MS at unit resolution (Liu et al., 2015; Frey et al., 2016). This neutron-encoded information can be taken advantage of for stable isotope labeling applications. Important examples of such applications include the biological production of internal standards with universally labeled (U)- $^{13}\text{C}$  (Neubauer et al., 2015) and  $^{13}\text{C}_3^{15}\text{N}_1$  (Basu et al., 2011; Snyder et al., 2014, 2015b) labeling as well as  $^{13}\text{C}$ -labeling of acyl-groups used in metabolic tracer studies (Worth et al., 2014), which are distinguishable by sufficiently high-resolution MS. Some pairs of acyl-CoAs, however, remain unresolved even by MS/HRMS. These include isobutyryl- and butyryl-CoA, warranting caution in the interpretation of data obtained for these two species with distinct cellular roles. To our knowledge, no reports of chromatographic separation for a number of these isomeric acyl-CoAs have been reported. As previously mentioned, neutral loss, or so-called “pseudo neutral loss” that relies on comparison of MS and MS/MS spectra in a trap-based analyzer, is a powerful tool for acyl-CoA analysis due to the distinct fragmentation of acyl-CoAs. Hybrid instruments, such as quadrupole/

Orbitrap platforms, can allow more effective neutral loss-type experiments but also a mixed targeted/untargeted experimental design, by using sensitive and selective MS/HRMS, combined with the untargeted full scan acquisition (Liu et al., 2015; Frey et al., 2016).

Quantitation of acyl-CoA analysis is also complicated by the notable impurity of some analytical standards. Contamination of commercial malonyl-CoA has been reported, as have high levels of contamination of succinyl-CoA (Neubauer et al., 2015). Additionally, degradation products, including CoASH or oxidized CoA, would be likely contaminations for any standard stored improperly or for long periods of time. To adjust for complicated bioanalytical procedures, including extraction, purification, and LC-MS analysis, internal standards for acyl-CoAs range from the use of non-CoA surrogate standards, to low-abundance or non-natural acyl-CoAs, to stable isotope labeled analogs of one or more acyl-CoAs (Ciccimaro and Blair, 2010). Heptadecanoyl-CoA has been commonly used as an internal standard for normalization of a wide range of acyl-CoAs (Demoz et al., 1993). Due to polarity differences resulting in unequal extraction efficiency, instability, and ion suppression by acyl-chain length, some concern may be warranted when analyzing a panel of acyl-CoAs with highly disparate acyl-groups (Yang and Drueckhamer, 2001; Liu et al., 2015; Frey et al., 2016). Commercially available stable isotope labeled acyl-CoAs include  $^{13}\text{C}$ -acyl labels, which, in the case of  $^{13}\text{C}_2$ -acetyl-CoA, may suffer from large isotopic overlap with the endogenous analyte. Furthermore, the degradation of an acyl-CoA internal standard with the stable isotope label on the acyl-chain, or no stable isotope label at all, would artificially inflate the free CoA in the sample. To circumvent this problem, and address providing an internal standard that matches the diversity of acyl-CoA analytes,  $^{13}\text{C}_3^{15}\text{N}_1$ -pantothenate has been previously used to biosynthetically generate a  $^{13}\text{C}_3^{15}\text{N}_1$ -acyl-CoA library for short-,

medium-, and long-chain acyl-CoAs (Basu et al., 2011; Snyder et al., 2015b). More recently, the method has been adapted to generate universally labeled (U)-<sup>13</sup>C-acyl-CoAs, which provides similar benefits to the <sup>13</sup>C<sub>3</sub><sup>15</sup>N<sub>1</sub>-acyl-CoA internal standard (Neubauer et al., 2015). Such rigorous quantitation and the plethora of analytical targets make acyl-CoA profiling ideal for biomarker studies in preclinical (Griffith et al., 2014), and even clinical settings (Worth et al., 2015).

## 22.4 Acyl-CoA Analysis for Current Drug Targets

Using acyl-CoAs as biomarkers for current drug targets has focused on the targeting of a small but critical subset of enzymes involving acyl-CoA metabolism. Acetyl-CoA carboxylase (ACC) catalyzes the irreversible conversion of acetyl-CoA to malonyl-CoA and is found in two forms in humans. The first isoform, ACC1, catalyzes the rate-limiting step in de novo lipogenesis and is widely expressed with especially high levels in the liver and adipose tissue. ACC2, the second isoform, is a mitochondrially associated protein that regulates fatty acid oxidation through inhibition of carnitine palmitoyltransferase-1-mediated fatty acid transport. This biology has led to targeting of ACC for diabetes and nonalcoholic fatty liver disease with pan-ACC or isoform-specific inhibitors. Specific measurement of ACC activity thus relies on quantification of acetyl- and malonyl-CoA levels, or isotopic incorporation assays. In vitro and animal pre-clinical studies used malonyl-CoA levels as a sensitive and specific end-point to steer development of ACC inhibitors through the preclinical pipeline, including cell-based measurements as well as the direct measurement of the dose-response dependent reduction in malonyl-CoA in the target tissues of treated animals (Griffith et al., 2014).

The targeting of cancer metabolism has likewise used acyl-CoA analysis to steer drug development. ACC inhibition has been targeted in cancer cell growth. Tracking synthesis of malonyl-CoA (dependent on ACC) versus methylmalonyl-CoA (not dependent on ACC) allowed specific evaluation of ACC targeting for a small molecule candidate. Aside from evaluation of known candidates, LC-MS monitoring of acyl-CoAs can be adapted for screening purposes. Fatty acid synthase (FAS), a metabolic target of interest in metabolic diseases as well as cancer, is also responsible for de novo lipogenesis by catalyzing the enzymatic condensation of acyl chains from malonyl-CoA and acetyl-CoA. A screen for inhibition of FAS was developed for 96-well-based screening from cells in culture, capable of screening 40 compounds

with a 9-point dose-response curve in under a day (Hopcroft and Fisher, 2016).

Interest in targeting post-translational modifications (PTMs) of proteins, especially histones, may also benefit from acyl-CoA analysis. The acyl-chains of PTMs (e.g., acetyl- in acetylation) are derived from acyl-CoAs, in a concentration-dependent manner (Lee et al., 2014). Discerning the specificity of various lysine acetyltransferases may benefit from acyl-CoA profiling (Montgomery et al., 2015), as the description of the number of potential PTMs is rapidly increasing (Tan et al., 2011). Similar to the examples with ACC and FAS enzymes, assays, screens, and preclinical studies may benefit from acyl-CoA quantitation by LC-MS to determine optimum candidates for progression.

## 22.5 Acyl-CoAs as Biomarkers in Metabolic Disease

Other than use in optimizing compounds for discrete targets currently in development, acyl-CoA profiling can provide biomarkers for metabolic diseases. A notable number of serious in-born errors of acyl-CoA metabolism occur in humans caused by mutations or co-factor deficiencies, resulting in reduced enzymatic expression, or expression of sub- or nonfunctional metabolic enzymes (Bennett, 2010). These include disorders of fatty acid oxidation and amino acid metabolism such as propionic academia resulting from mutations in either of the subunits encoding propionyl-CoA carboxylase, which leads to potentially toxic accumulation of metabolites. Some, but not all, of these disorders can be detected at birth with high sensitivity and specificity using well-established and cost-effective screening strategies of acyl-carnitines from blood spots; metabolic crisis can be prevented through early and stringent dietary restrictions (Boyle et al., 2014). This primary screening for these diseases is accomplished by MS, usually LC-MS/MS, using heel stick blood taken at birth or a few weeks after birth blotted onto specialized filter paper (blood spots). The actual analytes detected are acyl-carnitines, the circulating carriers for acyl-groups. The acyl-carnitines reflect the acyl-CoA pools within cells, but are at relatively high abundance in the blood, are excellent analytes for LC-MS/MS analysis due to a quaternary nitrogen and specific fragmentation, and can also reflect inborn errors of metabolism in carnitine metabolism. Unfortunately, some of these in-born errors affect essential metabolites that cannot be substituted in the diet, and thus, outcomes for patients remains poor. These disorders are informative as to the function of acyl-CoAs but also, with the promise of enzyme replacement therapies, gene therapy, and other novel therapeutic

approaches, may be eventually treatable. Such treatments could benefit from biomarkers of response, reflecting effectiveness and longevity of any therapies used. Carnitine profiles may be insufficiently sensitive as biomarkers, due to effects of diet or other confounders, but appropriate use of acyl-CoA profiling may directly quantify the underlying metabolic lesion (Palladino et al., 2012).

Previously limits in the detection capabilities for measurement of acyl-CoAs was prohibitive, but rapid progress in the field makes this a more attractive avenue for biomarker research. Aside from technological innovation, methodological innovations in stable isotope labeling and using surrogate biospecimens may help translate acyl-CoA analysis to clinical studies. A potentially powerful ex vivo approach using platelets as a surrogate tissue was developed and then applied to Friedreich's ataxia (FA), a disorder of iron-sulfur cluster metabolism (Basu et al., 2013). The relative rarity, pace of degeneration, and irreversibility of disease progression in FA make testing of therapeutics a major unaddressed challenge. By using a potentially treatment-reflective metabolic biomarker, the incorporation of stable isotope labels into acyl-CoAs in metabolically live platelets directly from patients (e.g., the incorporation of  $^{13}\text{C}$  isotopes from  $^{13}\text{C}_6$ -glucose into acetyl-CoA), inherent challenges in addressing normalization across patients and testing the early response to therapies may be overcome (Worth et al., 2015).

## 22.6 The Involvement of Acyl-CoAs in Drug Metabolism

Acyl-CoAs play an underappreciated and often ignored role in drug metabolism. However, as recent studies on quite well-studied parent drugs have revealed, acyl-CoA

metabolites may have been overlooked (Olsen et al., 2005, 2007). Thus, these studies warrant consideration of acyl-CoA formation as a route of biotransformation. First, based on studies with carboxylic acid-containing drugs known to form acyl-CoA metabolites, the drug-CoA conjugates were 70–100 times more reactive toward glutathione to form acyl-SG adducts than the more typically studied acyl glucuronide (Sidenius et al., 2004). This is important because of concerns of idiopathic hepatotoxicity as well as idiopathic immune reactions that are thought to be more likely to do with endogenous protein modification (Lassila et al., 2015). Secondly, even in the absence of detection of a stable acyl-CoA, conjugates dependent on acyl-CoA metabolism are often detected, such as taurine, glycine, and carnitine conjugation. For a limited number of therapeutics, acyl-CoA formation is already understood to play a role in the action of the drug as well as major toxicity (Yao et al., 1994). For example, the acyl-CoA metabolite serves to interconvert the R and S enantiomers of ibuprofen, which makes a relatively stable ibuprofenyl-CoA thioester (Tracy et al., 1993).

Another example of acyl-CoA forming small molecule therapeutics includes valproic acid (VPA), an effective anti-epileptic drug with dose-related hepatotoxicity and teratogenic potential. It undergoes extensive oxidative metabolism in humans, predominantly mediated by the formation of an acyl-CoA, as well as cytochrome P450 metabolism (Kassahun et al., 1994). In fact, one acyl-CoA-dependent metabolite, 2-n-propyl-2(E)-pentenoic acid, maintains anti-epileptic activity. Aside from the studies of the acyl-CoA-dependent and -independent metabolites of VPA, the impairment of mitochondrial  $\beta$ -oxidation is observed with VPA dosing (Silva et al., 2008). Thus, bioanalysis of acyl-CoAs in the context of VPA therapy, or of analogs of VPA in development, can not only reveal metabolism of the active drug but also provide a biomarker for the side effects of VPA.

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## 23

### Neurotransmitter Biomarkers

*Guodong Zhang*

*Bioanalytical and Biomarker Development, Shire, Lexington, MA, USA*

#### 23.1 Introduction

Neurotransmitters (NTs) are involved in a variety of regulatory systems such as stress and learning, and in the control of many processes of metabolism and the immune system (Bergquist et al., 2002). They can be divided into several compound groups that differ in chemical structures, such as amino acids (glutamic acid (Glu), aspartic acid, and glycine), catecholamines (dopamine (DA) and serotonin), acetylcholine (ACh), and neuropeptides (Kauppila et al., 2006). Concentrations of the NTs in the brain have been shown to relate to cognitive processes, such as learning and memory, as well as to various neurological disorders, such as schizophrenia, anxiety, epilepsy, and Alzheimer's and Parkinson's diseases (Mellon and Griffin, 2002; Tsai, 2000). Catecholamines, such as DA, norepinephrine (NE), and epinephrine (E), act as NTs at central and peripheral levels. DA is the most abundant one of the monoamine NTs and is found in non-neuronal tissues such as the gastrointestinal tract and the kidneys. Schizophrenia and Parkinson's disease have been linked to a deficient DA neurotransmission (Bicker et al., 2013; Rozet et al., 2006; Zeng et al., 2005). DA not only is the precursor of NE and E but also plays an important role as NT in the central nervous system (CNS). NE is primarily synthesized in the peripheral sympathetic nerve endings, where it functions as an NT, whereas E is predominantly synthesized in the adrenal gland (Braestrup et al., 1974), where it is stored in the specialized granules together with smaller amounts of NE. The substances 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) and Vanillylmandelic acid, 3-methoxy-4-hydroxymandelic acid (VMA) are the main metabolites of NE and E. Catecholamines act via dopaminergic and adrenergic receptors and are involved in a variety of regulatory systems. They take part in the regulation of the response to stress, psychomotor activity, emotional processes, learning, sleep, and memory (Durstewitz et al.,

1999; Python et al., 1996; Southwick et al., 1999). NE is known as the primary NT of postganglionic sympathetic adrenergic mechanisms, which are responsible for human cardiovascular control. The involvement of these NTs in multiple regulatory systems and metabolic processes supports their important use as biomarkers for the diagnosis, therapy, and prognosis of several neuroendocrine and cardiovascular disorders (Bergquist et al., 2002; Raggi et al., 2003). Glutamate and aspartate are the most abundant free amino acids in the brain and are classed as excitatory NTs that are released in a  $\text{Ca}^{2+}$ -dependent manner.  $\gamma$ -aminobutyric acid (GABA) is the main inhibitory NT in the CNS. As many as 10–40% of nerve terminals in the cerebral cortex, hippocampus, and substantia nigra may use GABA as an NT (Shah et al., 2002). In addition, GABA plays a rather important role in the spinal cord as revealed by its presence throughout the spinal gray matter except for motor regions. GABA is a key transmitter for inhibitory control mechanisms in the brain. Therefore, the main implication of GABA is in the pathophysiology of epilepsy, anxiety, and schizophrenia. Glycine is the second major inhibitory NT in the CNS. Its role as a NT has been most conclusively established in inhibitory interneurons in the spinal cord. Amino acid NTs represent a major class of biochemicals that are involved in neuronal communications at synapses in the CNS. ACh, one of the NTs released from cholinergic neurons in the CNS, plays an important role in sleep regulation, learning and memory, cognitive functions, and pathology of neurological disorders such as Alzheimer's disease (Wessler et al., 1998). A decrease in ACh levels in the brain is well established as a contributor to memory dysfunction in Alzheimer's disease. Therefore, drugs that increase the release of ACh are being explored as potential treatments for the cognitive symptoms of Alzheimer's disease. Histamine (HA), released from histaminergic neurons, has also been demonstrated to be an important NT regulating many

of the same brain functions as ACh. Central HA is metabolized to its two major metabolites (*tele*-methylhistamine (*t*-MHA) and *tele*-methylimidazole acetic acid (*t*-MIAA)). Thus, the concentrations of HA, *t*-MHA, and *t*-MIAA in the cerebrospinal fluid (CSF) have been used as indices of brain histaminergic activity. Histaminergic neurons are exclusively located in the tuberomammillary nucleus of the posterior hypothalamus, from where they project to practically all brain regions. HA is involved in the regulation of important neurophysiological functions, such as locomotor activity, sleep–wake cycle, attention, cognition, memory, and stress responses (Brown et al., 2001). Changes in brain HA levels have been observed in many neurological disorders, including multiple sclerosis, Alzheimer’s disease, febrile convulsions, narcolepsy, and hypersomnia (Dauvilliers et al., 2012; Wang et al., 2013). The concentrations of HA in CSF may be regarded as an indicator for central histaminergic activity. This makes the detection of CSF HA levels of potentially great significance for physiological research as well as for the clinic. Due to the presence of a wide range functions, NTs in the CNS and in periphery that they are involved in, a development of reliable and sensitive bioanalytical assays is essential.

## 23.2 Chromatographic Platforms of Biological Measurement for Neurotransmitters

### 23.2.1 Challenges for Neurotransmitter Measurement

Quantitation of NTs in routine laboratory has triggered an ongoing debate about which technique offers more advantages. Catecholamines exist in biological samples at extremely low concentrations, demanding highly sensitive and selective bioanalytical methods. Moreover, they are chemically unstable, prone to spontaneous oxidation, and decompose easily at high pH (Ji et al., 2008; Rosano et al., 1991; Zhang et al., 2012). Several challenges exist for the measurement of ACh, HA, *t*-MHA, and *t*-MIAA in biological samples. In particular, due to its rapid conversion to acetate and choline by acetylcholinesterase (AChE), ACh is biologically unstable in CSF with estimated half-life of 1–2 ms at the synaptic cleft. In addition, catecholamines, amino acids, ACh, HA, *t*-MHA, and *t*-MIAA are polar compounds with low molecular weights, which make it difficult for these analytes to be retained on conventional reversed-phase liquid chromatography (LC) columns. It is also challenging to chromatographically separate these analytes from the inorganic salts and endogenous compounds present in biological samples to allow for the reliable measurement

of individual NT concentrations. Hence, highly sensitive and selective analytical methods are required to perform these measurements (Zhang et al., 2011).

### 23.2.2 LBA, LC, GC, and CE

Ligand binding assay (LBA) including radioenzymatic assay (REA), radioimmunoassay (RIA), and enzyme immunoassay (EIA) is employed to measure NTs in biological samples such as blood, urine, and brain tissue. Since REA, RIA, and EIA are based upon the specific reactions between antigen and antibody, the key step for developing an optimal protocol for measuring NT concentrations is the production of a potent and specific monoclonal or polyclonal antibody against analytes. Because NTs are small molecules without high immunogenic potency, it is relatively difficult to produce anti-NT antibodies with high specificity and high sensitivity. These methods are generally struggling with the interference problems caused by NT cross-reacting with other compounds (Oguri and Yoneya, 2002; Wang et al., 2013).

In the past several decades, the most common technique used for quantifying and profiling NTs is reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV), fluorescence (FL) (Yoshitake et al., 2004, 2006), or electrochemical (EC) detection (Cooper et al., 1994; Raggi et al., 1999). With HPLC, the sample extract is analyzed using the high-pressure output of liquid as the mobile phase, small particle size sorbent as the stationary phase. Ultra-performance liquid chromatography (UPLC), using a principle similar to HPLC, yields a significant improvement in the rapid separation of analytes via its smaller particle-size stationary phase and ultra-high pressure pumps. It is therefore better suited for NT separation than conventional HPLC. Characteristic features of most HA, DA, and catecholamines are their oxidation vulnerability and their native fluorescence properties, which explain the long history of conventional HPLC detection methods. HPLC-EC is still considered as the method of choice mainly because of the low cost and sensitivity.

From the 1970s, gas chromatography (GC) utilizing capillary columns linked with MS was introduced and applied to identify and quantify NTs and their metabolites present in the clinical samples (Anthony et al., 1970). Serotonin, 5-hydroxyindole-3-acetic acid (5-HIAA), and other tryptophan metabolites could be quantified by using GC-MS after derivatization (Markey et al., 1991). However, GC requires chemical derivatization as well as tremendous skills. In addition, complex matrix effects and sample carryover raise severe concerns for the results.

Recently, capillary electrophoresis (CE) including capillary zone electrophoresis (CZE) and capillary

electrochromatography (CEC) has become an attractive alternative. CZE and CEC methods work well with small volumes of sample that can be separated more quickly and with better specificity compared with some classic chromatographic methods. However, they are rarely applied for NT measurement in biological samples, possibly due to their lower sensitivity and the lesser precision of the sample migration time.

### 23.2.3 LC–MS/MS

Owing to the varied efficiency and time consumption of derivatization, a simplified sample preparation using liquid chromatography coupled with electrospray tandem mass spectrometry (LC–ESI–MS/MS) was widely employed to measure NTs and its metabolites in the biological samples. Mass spectrometry is superior to the other detection methods as it has the advantage of giving additional structural information about the analytes and being remarkably sensitive (Kauppila et al., 2006; Kushnir et al., 2002; Qu et al., 2002).

LC–MS/MS enables the use of sophisticated sample pretreatment techniques and automation of the whole process by online coupling of the separate techniques. To correct for losses during sample pretreatment, analyte separation, and detection, stable isotopes of analytes are used as internal standards with mass spectrometry. Non-mass spectrometric methods cannot use these isotopes, because isotopes, in general, co-elute chromatographically with the analytes of interest and can only be separately detected from the analyte based on mass differences. LC–MS/MS combines the physical separation capabilities of HPLC with the high analytical sensitivity, specificity, and accuracy of mass spectrometric detection. In recent years, continuous improvement of LC–MS/MS equipment in performance has been observed. Due to its superior specificity, shorter runtimes, and less laborious sample preparation, LC–MS/MS methods replace more and more of the previously described HPLC and GC–MS techniques to measure NTs in biological samples (Maurer, 2006). Profiling of those samples also allows evaluation of metabolic pathways, which might simplify diagnosis and is more cost-effective. Therefore, the most used detection method for the bioanalysis of NTs these days is mass spectrometry (Hows et al., 2002; Li et al., 2000; Song et al., 2005; Zhu et al., 2000). A challenge with LC–MS/MS quantitation of biological samples is the interindividual variation in body fluids composition, which can result in alteration of ionization efficiency. These matrix effects are compound-specific and need to be evaluated for assay validation per FDA bioanalytical validation guidance (FDA, 2001, 2013). In this chapter, an overview of the most commonly used and cutting-edge LC–MS/MS assays for the NT measurement will be

described along with techniques for the extraction, matrix stability, derivatization, and HPLC chromatographic conditions.

## 23.3 Bioanalytical Methodologies

### 23.3.1 Sample Preparation Strategies

Sample cleanup and extraction play key roles for NT bioassays. Nowadays, solid-phase extraction (SPE) is most widely used for the extraction and pre-concentration of NTs with regard to the high extraction efficiency and selectivity (Cakal et al., 2011). Indeed SPE is regarded as an effective pre-concentration method that not only removes the interfering substances but also concentrates the analytes of interest to detectable levels. Briefly, it uses the same principles as chromatography, except that the majority of sample components are retained on the stationary phase upon sample loading. After washing the SPE cartridge and removing the unwanted low-affinity binding compounds, NTs are eluted from the cartridge using a solution with adequate elution strength. In order to achieve optimal recovery and minimal matrix effects, different loading, washing, and elution procedures such as change of pH, organic solvent composition, ionic strength, and elution volume/speed must be investigated (Raggi et al., 1999). Several types of cartridges can be utilized for SPE procedures, such as hydrophilic–lipophilic balance (HLB) cartridges (Raggi et al., 1999), strong or weak cation exchange cartridges (Raggi et al., 2003), alumina cartridges (Zhang et al., 2012), and phenylboronic acid (PBA) cartridges (Rozet et al., 2006). In particular, PBA cartridges and alumina are utilized to extract catecholamines. PBA can serve as an immobilized affinity ligand for online SPE and may be also applied in a 96-well cartridge and incorporated in an automated system that facilitated the overall procedure, reduced the analysis time, and enhanced the reproducibility of extraction method. Alumina (aluminum oxide) was applied to extract catecholamines from biological fluids. It has the ability to form cyclic complexes with the dihydroxyphenyl structure of catecholamines, which allows the isolation of all compounds that possess the catechol moiety from those that do not have that chemical group (Bergquist et al., 2002). Protein precipitation (PPT) was utilized to extract DA, NE, 5-hydroxytryptamine (5-HT), 5-HIAA, and GABA from plasma (Cai et al., 2010). 5-HT, DA, HA, *t*-MHA, L-DOPA, and 5-HIAA were isolated by perchloric acid or 0.5M formic acid from brain tissue or plasma. Ascorbic acid, 1,4-dithiothreitol, or sodium metabisulfite is utilized as preservative to prevent oxidation of the analytes (Wang et al., 2013; Yoshitake et al., 2004; Zhu et al., 2011) (Table 23.1).

**Table 23.1** LC–MS/MS techniques for the quantitation of NTs in biological samples.

Analytes	Biological matrices	Sample preparation	Column	Chromatography	Derivatization reagent	Calibration range	Reference
E and NE	Human plasma	SPE [Alumina B]	Onyx monolith C <sub>18</sub>	RP	Acetaldehyde- <i>d</i> <sub>4</sub>	0.0005–0.5; 0.02–2 ng/mL	Zhang et al. (2012)
DA; NE; E; Glu; GABA; 5-HT	Rat brain	Homogenization	ACE C <sub>18</sub>	RP	No	0.025–2.5 ng/mL	Zhu et al. (2011)
DA; DOPAC; HVA; NE; VMA; MHPG; 5-HT; 5-HIAA; Glu; GABA	Human plasma	PPT	C <sub>18</sub>	RP	Dansyl chloride	1.1–326; 1.0–297; 0.9–274; 1.0–296; 0.8–252; 0.9–271; 0.9–284; 0.9–262 pmol/mL; 9.1–2700; 0.2–58.2 nmol/mL	Cai et al. (2010)
HA; <i>t</i> -MHA	Human CSF	Direct derivatization	BEH C <sub>18</sub>	RP	4-BBS	50–5000 pM	Croyal et al. (2011)
DA; NE; 5-HT; NM	Rat CSF	Direct derivatization	BEH phenyl column	RP	Acetaldehyde- <i>d</i> <sub>4</sub>	0.01–1 ng/mL	Ji et al. (2008)
ACh; HA; <i>t</i> -MHA; <i>t</i> -MIAA	Rat CSF	PPT	Kinetex HILIC	HILIC	No	0.025–5; 0.05–10; 0.05–10; 0.05–10 ng/mL	Zhang et al. (2011)
Glu; GABA; choline; ACh; DA; 5-HIAA; serotonin; DOPAC; HVA	Rat brain	0.1M formic acid homogenization	BEH C <sub>18</sub>	HFBA ion-pair RP	No	2–200; 20–4000; 20–4000; 20–4000; 20–4000; 20–4000; 20–4000; 5–200; 20–4000 ng/mL	González et al. (2011)
NMN; MN	Human plasma	PPT	Hypercarb PGC	Mixed mode cation exchange and ion-pair RP	No	0.013–0.95; 0.006–0.46 ng/mL	He and Kozak (2012)
NMN; MN	Human plasma	SPE	Atlantis HILIC	HILIC	No	0.018–18.32; 0.019–19.72 ng/mL	Petteys et al. (2012)
NMN; MN	Human urine	Mixed mode cation exchange SPE	PFP propyl	RP	No	4.58–1832; 4.93–1972 ng/mL	Clark and Frank (2011)
NMN; MN	Human urine	SPE	Atlantis T3 C <sub>18</sub>	RP	No	0.3–14.6; 0.2–27.4 nmol/mL	Gabler et al. (2011)
NMN; MN	Human plasma	Ion-pair SPE	Hypercarb PGC	RP	No	18–989; 7.2–487 pg/mL	He et al. (2011)
DA; DOPAC; HVA; 3-MT	Rat CSF	Freeze-drying	Gemini C <sub>18</sub>	RP	No	0.05–50 ng/mL	Syslová et al. (2011)
DA; NE; E	Human urine	PBA affinity SPE	PFP propyl	RP	No	1.83–1470; 2.70–846; 0.27–2748 ng/mL	de Jong et al. (2010)
NE; E	Rat plasma	PPT	BEH phenyl	RP	Acetaldehyde- <i>d</i> <sub>4</sub>	0.05–25 ng/mL	Ji et al. (2010)

NMN; MN; 3-MT	Human plasma	SPE	Atlantis HILIC	HILIC	No	0.018–4.21; 0.02–4.53; 0.017–3.85 ng/mL	Peaston et al. (2010)
DA; DOPAC; HVA; NMN; 3-MT; 5-HT; 5-HIAA	Rat brain dialysate	Direct injection	Discovery HS F5	RP	No	0.12–3.02; 30.3–168; 12.8–182 ng/mL	Uutela et al. (2009)
ACh; choline	Rat or mouse brain	Ringer's solution salt extraction	HILIC	HILIC	No	0.1–50; 100–3500 nM	Uutela et al. (2005)
L-DOPA; DA; NE; E; NMN; MN; 3-MT	Rat adrenal gland	PPT	Kromasil cyano	HILIC	No	20–3000 ng/mL for L-DOPA/MN; 20–10,000 ng/mL for DA/NE/E/3-MT/NMN	Gu et al. (2008)
DA; NE; E	Human urine	LLE	Allure basix	HILIC	No	0–7000; 0–600; 0–380 ng/mL	Kushnir et al. (2002)
L-DOPA; DA	Rat plasma	Alumina SPE	ODS	RP	No	2.5–1000 ng/mL	Li et al. (2000)
NE	Rat urine	Alumina SPE	Zorbax Rx-C <sub>8</sub>	Ion-pair RP	No	5–500 ng/mL	Neubecker et al. (1998)

### 23.3.2 Sensitivity and Chromatography

#### Enhancement by Chemical Derivatization Using LC-MS/MS

The sensitivity achieved by an LC-MS/MS platform depends on the efficiency of NTs' ionization efficiency, which, in turn, can be affected by the characteristics of the analyte, chromatographic variables, and matrix effects (Kushnir et al., 2002). For instance, the sensitivity of electrospray ionization is not favored by highly polar and small molecules, such as NTs. Very polar and small analytes are more susceptible to ion suppression (Annesley, 2003). This emphasizes the importance of sample pretreatment prior to LC-MS/MS analysis. Many interfering peaks resulting in low sensitivity and poor specificity could occur in the low-*m/z* region when biological samples are analyzed by mass spectral procedures. In one study, MS detection of one sample had to be carried out twice in both positive and negative ion modes separately to achieve respective analysis of NTs and their acidic metabolites (Gu et al., 2008). Thus, it is beneficial to derivatize NTs and their metabolites for the purpose of increasing mass, introducing an electrophilic group and enhancing chromatographic separation as well (Table 23.1).

Diethyl labeling of amino group of monoamine NTs such as NE, E, DA, 5-HT, and normetanephrine (NM) affords 20–100 times of increased sensitivity combined with UPLC-MS/MS. This could result from improved fragmentation patterns, increased hydrophobicity, concomitantly increased ionization efficiency in ESI MS and MS/MS analysis, and reduced matrix interference. Diethylation labeling employs a commercially available reagent, acetaldehyde-*d*<sub>4</sub>, to label the amine groups on the monoamines via reductive amination. It is also simple, fast (~25-min reaction time), specific, and quantitative under mild reaction conditions (Ji et al., 2008; Zhang et al., 2012).

Dansyl chloride as a derivative agent has been used to improve electrospray ionization (ESI) signal intensity for ultra-trace analysis of monoamine NTs. An LC-MS/MS assay utilized dansyl chloride as derivatization reagent for the simultaneous measurement of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), vanillylmandelic acid, 3-Methoxy-4-hydroxymandelic acid (VMA), 3-methoxy-4-hydroxyphenylethylene-glycol (MHPG), 5-HT, 5-HIAA, Glu, GABA, and NE in human plasma. This method is highly sensitive and selective and can be clinically applied as a biochemical index for certain disease and the monitoring of drug therapy (Cai et al., 2010).

An ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) assay involving derivatization of primary amines in HA and *t*-MHA with 4-bromobenzenesulfonyl chloride (4-BBS) was described for the simultaneous analysis of HA and its

major metabolite *t*-MHA from human CSF samples. Lower limit of quantitation (LLOQ) was 5.5 pg/mL for HA and 6.25 pg/mL for *t*-MHA. The sensitive mass spectrometric detection of bromine-containing compounds leads to strong isotopic signals with duplicate peaks corresponding to the two isotopes of the halogen. Indeed, the LOD (12.5 pM for each amine) was 200 times lower than traditional LC-MS/MS method. The conditions of the derivatization reaction of both amines and their chemically synthesized IS were optimized to favor the selectively derivatization on the primary amine group (temperature and kinetic optimization), leaving the imidazole nucleus intact in the case of HA derivatization, thereby avoiding the formation of multiple derivatives with variable stability and poor reproducibility. The chemical derivatization reaction is simple, by using a commercially available reagent, and is fast (<10 min reaction time), specific, and quantitative under mild reaction conditions. As a result, a nearly quantitative derivatization was found with reaction yields of 103 and 99% for HA and *t*-MHA, respectively (Croyal et al., 2011).

### 23.3.3 Chromatographic Strategies for LC-MS/MS Assays

As mentioned previously, NTs are low molecular weight molecules with a very polar nature. Commonly used reversed-phase C18 or octadecylsilane (ODS) columns demonstrate the fast elution of polar compounds and the relatively short run time. There is a danger of an excessively fast elution and poor peak resolution, due to the reduced retention of polar compounds on the hydrophobic stationary phase. To overpass this limitation, some recent columns, such as the Atlantis T3 column, have C18-alkyl phases bonded at ligands with lower density than those used in other columns, promoting the retention of polar compounds (Gabler et al., 2011). Their lower ligand density allows a quicker access of analytes to the pore structure of the material, where the retention occurs for the majority of the compounds, and, consequently, the retention increases. Another possibility is the use of C18 monolithic columns (Zhang et al., 2012). These columns have porous rods of silica instead of individual silica particles and demonstrated superior chromatographic separation and peak shape for E and NE than other tested columns. Other less frequently used columns are composed by base-deactivated silica (BDS) and C30 stationary phases. BDS columns overcome some limitations of conventional silica stationary phases, such as peak-tailing, by reducing unwanted silanol interactions. The only method developed for NTs using a C30 column allowed the simultaneous measurement of L-DOPA, DA, NE, E, and

DHPG, interestingly, without requiring an ion-pairing reagent (Machida et al., 2006).

Another alternative has been the utilization of ion-pairing reagents to increase the retention time of NTs and to provide separation from various matrix components that elute at the solvent front. However, if MS is used as detection system, nonvolatile ion pairs should provoke ion suppression, and hence, volatile ion-pairing reagents such as heptafluorobutyric acid (HFBA) should be preferred (Eckstein et al., 2008; Keski-Rahkonen et al., 2007; Zhu et al., 2000). When mobile phase of 0.01% (v/v) formic acid was used, some compounds such as Glu, ACh, GABA, and choline were poorly retained on the C<sub>18</sub> column. This problem could be solved by using HFBA, which increases the retention time of the analytes and improves the peak shape of the compounds. Several concentrations of HFBA were tested (from 1 to 10 mM in the aqueous phase), and it was noted that when high concentration of HFBA was used, the retention time of the compounds was longer. However, when the concentration of HFBA was higher than 2 mM, two peaks were observed for ACh. Bearing in mind this fact, 1 mM of HFBA in the aqueous phase was used for further experiments (González et al., 2011).

The porous graphitic carbon (PGC) columns have been recently used for accurately quantifying L-DOPA, DA, NE, and E in human plasma (He et al., 2011), and in porcine brain (Tornkvist et al., 2004). They have a particular retention mechanism based on the interaction between polar analytes and the polarizable surface of graphite and are therefore appropriate for the separation of highly polar compounds with similar structures such as the catecholamines. PGC columns are normally coupled with MS detection, probably as a strategy to avoid ion-pairing reagents in the mobile phase.

Cyano derivatives groups in the stationary phase, such as Kromasil Cyano column (Gu et al., 2008), produced good peak shapes for DA, NE, and E without ion-pairing reagents (Kushnir et al., 2002). Separation of DA, L-DOPA, E, and NE was obtained on a cyano analytical column (150 mm × 2.1 mm (i.d.), particle diameter of 5 μm, pore diameter of 80 Å) by a mobile phase consisting of 60% (v/v) acetonitrile and 40% (v/v) water adjusted with formic acid to pH 3.0 (Gu et al., 2008). Chromatographic separation was followed by positive ion electrospray ionization tandem mass spectrometry in the multiple reaction monitoring modes.

An interesting option to reversed-phase and traditional normal phase separations is hydrophilic interaction liquid chromatography (HILIC), also known as aqueous normal phase chromatography. HILIC columns have a water-rich layer over the polar

stationary phase. Analytes will therefore interact with the hydrophilic environment provided by the water-rich layer by hydrogen and electrostatic bonding. These columns are frequently coupled with MS detection. The application of HILIC columns followed by mass spectrometric detection was demonstrated to achieve rapid and complete separation of catecholamines and selected metabolites (de Jong et al., 2007). The column selection for the retention of ACh, HA, t-MHA, and t-MIAA proved to be challenging due to the highly polar and hydrophilic nature of these molecules. A number of chromatographic procedures have been previously reported for the analysis of ACh in microdialysis samples, including reversed-phase, ion exchange, or ion-pair chromatography. However, the sensitivities of these methods were poor because of the highly aqueous mobile phases or the high concentrations of salt and mobile phase additives used. Although ion pairing can provide improved retention, ion-pairing agents impede quantification due to the potential for ion suppression and MS source pollution. An HILIC–MS/MS method was developed using Kinetex fused-core HILIC Silica (100 mm × 2.1 mm, 2.6 μm). This method provided efficient resolution for ACh, HA, t-MHA, and t-MIAA with a run time of 7 min under optimized LC gradient elution conditions at a flow rate of 0.2 mL/min (Zhang et al., 2011).

### 23.3.4 NTs Stability and Sample Collection

The quantification of NTs in biological samples usually requires elaborate and labor-intensive cleanup procedures due not only to their low physiological concentrations and chemical instability such as ACh and catecholamines but also to the complexity of biological matrices and to the presence of potential interfering compounds (Gu et al., 2008). A widely used technique for collecting samples in brain is microdialysis, where a physiological perfusion fluid is pumped through a dialysis membrane that is surgically implanted into the brain.

Although *in vivo* microdialysis is used in preclinical studies to assess the release of NTs, the translation of these findings from preclinical to clinical studies has yet to be demonstrated. When no enzyme inhibitors were added to collection vials, the ACh concentrations were below the lower limit of quantification (BLQ) in human CSF, suggesting that ACh may undergo significant degradation from sample collection through analysis (Zhang et al., 2011). The stability of ACh, HA, t-MHA, and t-MIAA in CSF was explored to maintain sample integrity via enzymatic inhibitors. During incubation in fresh rat CSF, the *in vitro* kinetic data indicated that exogenously spiked ACh rapidly disappeared with an *in vitro*

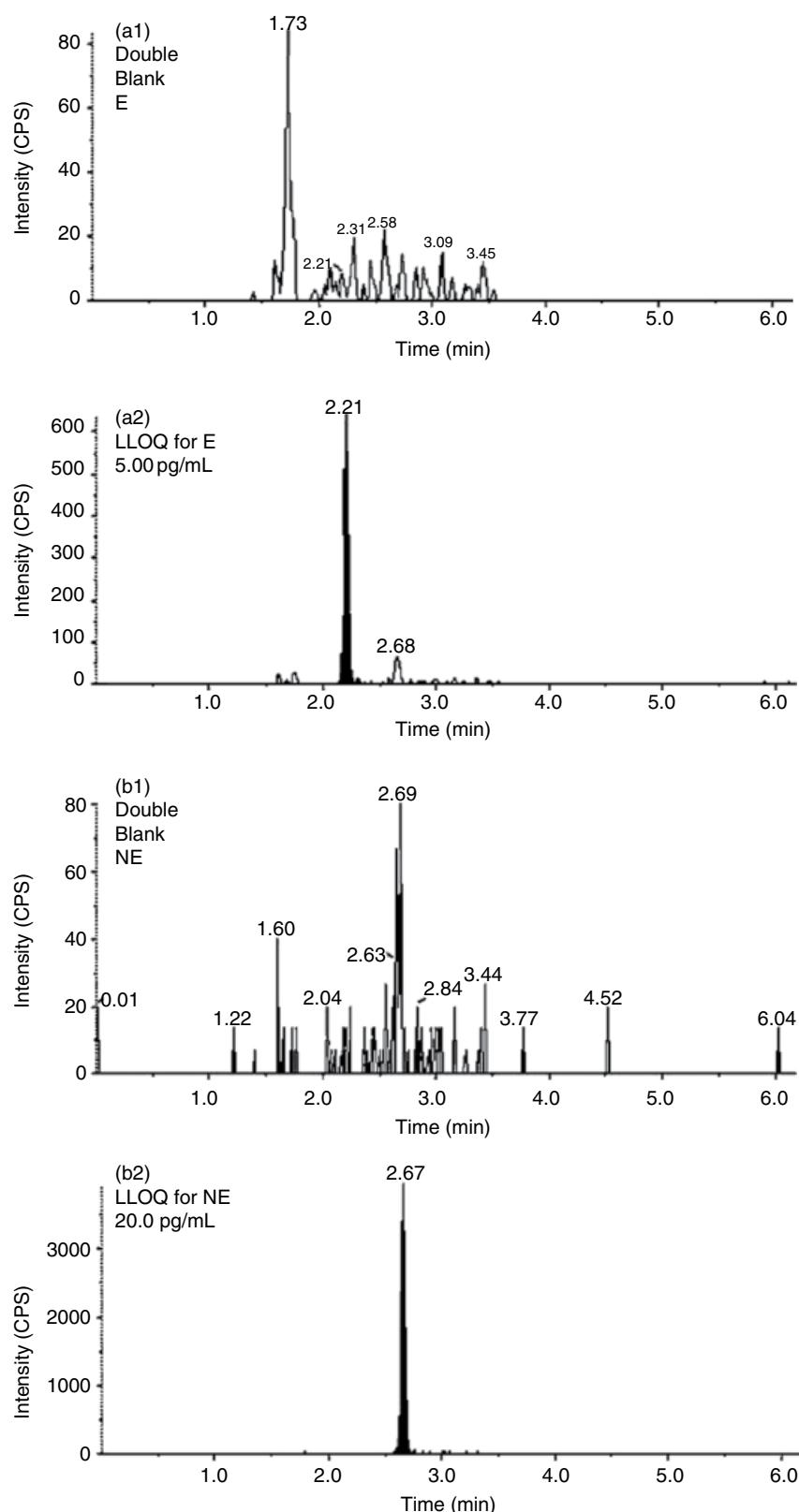
half-life of  $1.9 \pm 0.4$  min. In contrast, spiked ACh was stable for up to 60 min at room temperature when incubated in rat CSF pretreated with serine, which was reported to be a potent noncompetitive inhibitor of AChE when preincubated with the enzyme for as short a time of 6 min. Hence, the observed decreases in ACh concentration in rat CSF samples over the incubation period were linked to enzyme-related biological instability, as confirmed by the fact that the enzyme AChE exists in CSF and that the half-life of ACh was prolonged to more than 60 min in the presence of AChE inhibitor. Additionally, the *in vitro* half-lives of ACh in human and monkey CSF were determined as  $4.6 \pm 0.2$  min and  $8.9 \pm 0.4$  min, respectively, thus further confirming its instability in CSF samples. As a result, ACh cannot be measured in CSF under conditions commonly used for sampling due to its extremely short *in vitro* half-life. Thus, identifying and incorporating a stabilizing condition that will preserve the integrity of ACh is a key factor for ACh measurement in CSF. Three stabilizing agents (HCl and AChE inhibitors eserine and diisopropyl fluorophosphate (DFP)) were investigated for their ability to stabilize ACh. ACh concentrations were BLQ when samples were collected into untreated tubes, indicating that the flash-freezing alone cannot adequately prevent the ACh degradation processes. Although all three test conditions appeared to stabilize ACh at detectable levels, ACh was best stabilized by eserine-treated tubes as indicated by an approximately 1.5-fold greater concentration when compared with HCl- or DFP-treated tubes, making it the preferred collection tube. Finally, an eserine concentration series was utilized to investigate the concentration-dependence of the inhibition of degradation of ACh in rat CSF. The results demonstrated that the inhibitory effects of eserine were independent of its final concentration in the range of 25–200 mM. This is likely due to the fact that eserine concentrations at and above 25 mM (Stein and Lewis, 1969) can completely inhibit AChE activity, resulting in a maximal stabilization of ACh levels in rat CSF at all concentrations tested. In summary, 25 mM eserine was chosen to stabilize samples to enable reliable ACh measurements (Zhang et al., 2011).

Owing to thermal instability of catecholamine, there are additional strategies to be used for this type of analytes in order to prevent sample instability issues. The most significant variables that affect compound stability during sample storage are the environment temperature, sample pH, freeze/thaw times, storage time, and with/without preservatives. The goal of maintaining sample integrity for NTs such as catecholamines can be achieved by lowering the pH of the samples, particularly for long-term storage (Roberts et al., 2010). Preservatives such as hydrochloric acid, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), and sodium metabisulfite

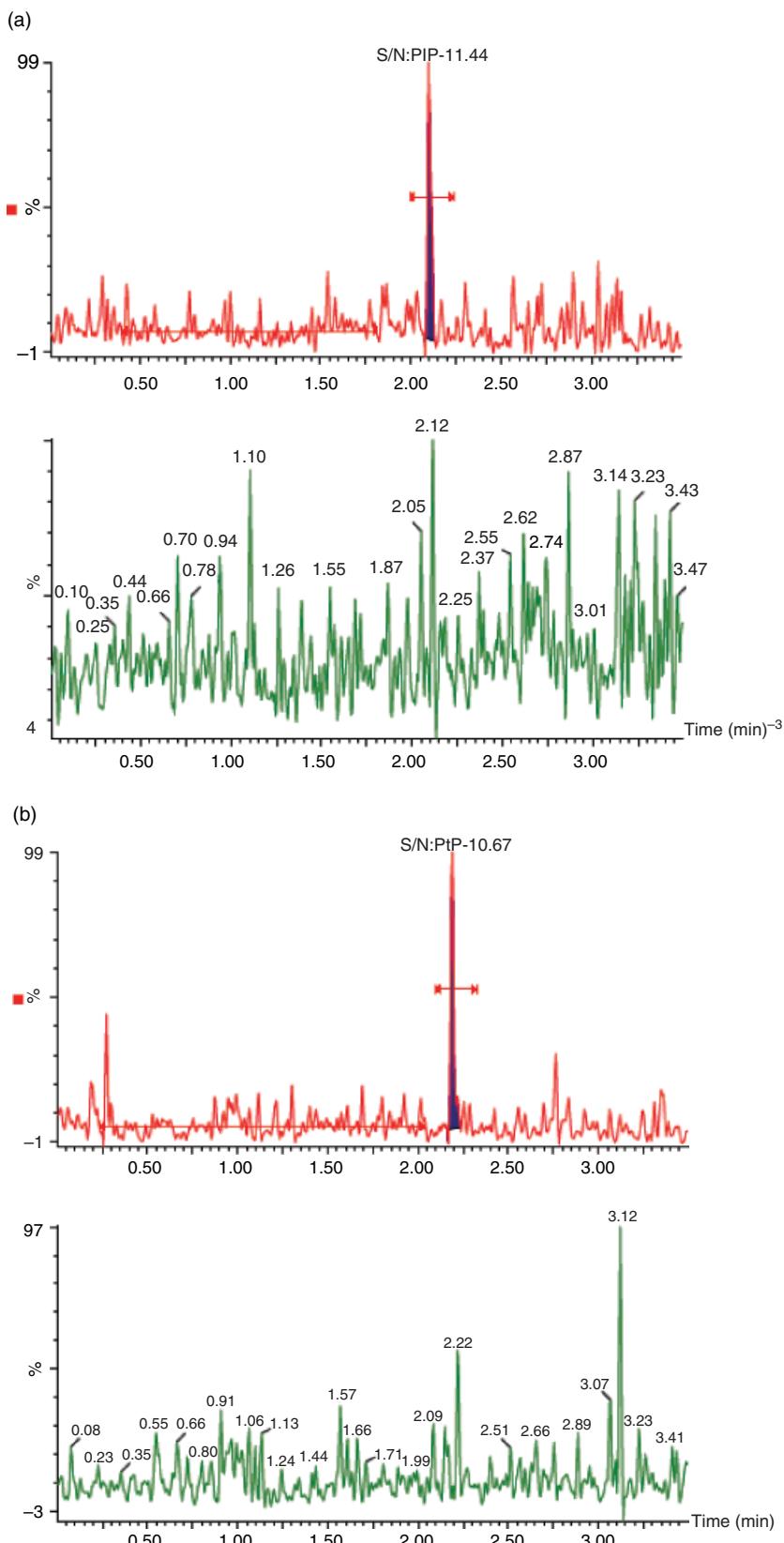
are frequently used (Tsunoda, 2006; Wolthers et al., 1997). The stability studies of catecholamines were mainly conducted on plasma, urine, and CSF samples with few data found concerning tissue samples. Considering urine samples, it is recommended that the pH of urine should be less than 4.0 or 3.5. Indeed, urine acidification to pH 4.0 is regarded as an effective mean to prevent catecholamine decomposition. Some authors suggest that urine ought to be collected into vials already containing acid in order to enhance stability (Roberts et al., 2010). An alternative would be adding antioxidant agents during sample collection and acidifying only after receiving the sample (Willemsen et al., 2007). It was reported that adding EDTA and sodium metabisulfite to the collection vessel was enough to stabilize catecholamines in 24-h urine samples for 2 weeks at  $-20^{\circ}\text{C}$ . It is important to highlight that urine acidification to pH values below 2.0 must be avoided because the hydrolysis of the conjugated forms of catecholamines may occur and increase free catecholamine concentrations. Acidification is considered the best method for prolonged preservation of urine samples for catecholamine quantification (Boomsma et al., 1993), independently of the acid that is used for that purpose. Hydrochloric acid, acetic acid, and sulfuric acid are available options, and hydrochloric acid is the most frequently used.

E and NE stability was systematically assessed in plasma and whole blood (Zhang et al., 2012) in order to establish proper sample handling conditions for clinical research sites. E and NE were not stable at room temperature within 4 h in human blood without stabilizers (37.0 and 29.4% of nominal, respectively). However, E and NE in untreated human blood were much more stable at  $4^{\circ}\text{C}$  (up to 2 h). Plasma E and NE levels reduced to 64.8 and 85.0% of nominal, respectively, at room temperature for 24 h without stabilizers, while they remained stable for at least 24 h when treated with sodium metabisulfite (6.34 mg/mL in matrix) or citric acid and ascorbic acid solution (25 mg/mL of citric acid and 2 mg/mL of ascorbic acid in matrix). From the stability assessment, it was determined that it is not necessary to add the stabilizers to blood and plasma immediately following collection or processing. It is recommended that upon collection whole-blood samples be kept at  $4^{\circ}\text{C}$  and centrifuged within 60 min (also at  $4^{\circ}\text{C}$ ). The separated plasma should then be frozen immediately at  $-80^{\circ}\text{C}$  and stored until sample analysis.

There are several protocols for the collection of catecholamines from blood samples, but it is consensual that blood should be centrifuged within 1 h after collection, in order to separate plasma as soon as possible and consequently minimize the loss of catecholamines. Many procedures perform blood sample centrifugation



**Figure 23.1** Representative LC-MS/MS chromatograms of LLOQ (5.00 pg/mL for E and 20.0 pg/mL for NE) in artificial plasma (a1) blank for E, (b1) blank for NE, (a2) 5.00 pg/mL of E spiked in artificial plasma, and (b2) 20.0 pg/mL of NE spiked in artificial plasma. Source: Zhang et al. (2012). Reproduced with permission of Elsevier.



**Figure 23.2** Representative chromatogram of 4-BBS-derivatized HA (a) and t-MHA (b) at the LOD (12.5 pM) compared with an artificial blank CSF sample. Source: Croyal et al. (2011). Reproduced with permission of Elsevier.

at 4°C (Machida et al., 2006). Different antioxidants are usually added prior to centrifugation to minimize loss of catecholamines. Catecholamines in plasma samples are found to be stable for 6 weeks at -20°C and for 1 year at -80°C without antioxidant, and for 24 h at RT during three freeze-thaw cycles, and for 1 year at -80°C with antioxidant. For this reason, plasma should be stored at -80°C whenever possible and antioxidants preferably incorporated. Ideally, blood ought to be collected into tubes containing an anticoagulant and an antioxidant (Zhang et al., 2012), transported on ice, centrifuged at 4°C, and stored at -80°C. Plasma metanephrines (MNs) are not as susceptible to external influences as catecholamines and, hence, sample collection protocols are less strict (Peaston and Weinkove, 2004). CSF collection and storage must follow strict laboratorial procedures. The metabolites of the NTs are relatively stable in CSF, but blood contamination can lead to their oxidation if red blood cells hemolyze. Accordingly, samples contaminated with blood should be centrifuged as soon as possible before freezing. Interestingly, all metabolites are stable in frozen CSF samples at -70°C for 5 years, without the use of antioxidants (Hyland, 2008).

### 23.3.5 Case Studies

Measurement of endogenous E and NE in human plasma is very challenging due to lower endogenous concentrations as compared with animal plasma. An LC-MS/MS, in combination with alumina-based SPE and derivatization procedure, was validated for the measurement of E and NE in human plasma with acceptable intra- and inter-day accuracy and precision (Zhang et al., 2012). Sample was extracted with semi-automated alumina 96-well SPE cartridge. The resulting eluent was dried and derivatized using acetaldehyde-*d*<sub>4</sub>. The analytes were separated on a monolithic C18 column. Extraction efficiencies were >66% for E and NE. The LLOQ was 5.00 pg/mL for E and 20.0 pg/mL for NE in heat-treated human plasma (Figure 23.1).

A UPLC-MS/MS assay was developed for the simultaneous analysis of HA and its major metabolite *t*-MHA, from CSF samples (Croyal et al., 2011). The method involves

derivatization of primary amines with 4-BBS and subsequent analysis by LC-MS/MS in positive electrospray ionization. The separation of derivatized biogenic amines was achieved within 3.5 min on an Acquity BEH C18 column by elution with a linear gradient of acetonitrile/water/formic acid (0.1%) (Figure 23.2). The assay was linear in the concentration range of 50–5000 pM for each amine (5.5–555 pg/mL for HA and 6.25–625 pg/mL for *t*-MHA). For repeatability and precision determination, coefficients of variation (CVs) were less than 11.0% over the tested concentration ranges, within acceptance criteria. Thus, the developed method provides the rapid, easy, highly sensitive, and selective performance to quantify these amines in human CSF. No significant difference was found in the mean ± standard error levels of these amines between a group of narcoleptic patients (HA = 392 ± 64 pM, *t*-MHA = 2431 ± 461 pM, *n* = 7) and of neurological control subjects (HA = 402 ± 72 pM, *t*-MHA = 2209 ± 463 pM, *n* = 32).

## 23.4 Conclusion

LC-MS/MS is becoming the mainstream technique for NTs in biological samples (Table 23.1) due to its excellent sensitivity, specificity, and accuracy. There is a great variety of recent LC-MS/MS methodologies developed for the measurement of NTs, which combine optimized sample preparation, chemical derivatization, and chromatographic conditions. It enables more sensitive and specific measurement of NTs and the routine quantitation of NT biomarkers in low concentration ranges, with reproducibility, high throughput, and short run time to handle a large number of samples. A clearer understanding of the role that NTs and their metabolites play in certain diseases is important not only for diagnosis purposes but also for the research and development of new drugs. The wide interest of NTs in fluid samples, particularly plasma, CSF, and urine from human origin, is justified by their usefulness as practical diagnostic samples for NT-related pathologies. This chapter may be considered as an informative tool to support the development of new LC-MS/MS assays for quantification of NTs in biological samples as biomarkers.

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## 24

### Targeted Quantification of Carbohydrate Biomarkers Using LC–MS

Cong Wei and Hong Gao

*Drug Metabolism & Pharmacokinetics, Vertex Pharmaceuticals Incorporated, Boston, MA, USA*

#### 24.1 Introduction

Carbohydrates, a synonym of saccharide, are divided into four chemical groups: monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Carbohydrates are an important class of biomolecules that play critical roles in biological processes, such as protein trafficking, cell-to-cell communication, and immune responses, as well as pathogenic processes in which the abnormality of carbohydrates could be associated with numerous diseases including cancer, dementia, and autoimmune disorders (Dennis et al., 2009; Dove, 2001; Yang et al., 2015). Carbohydrates often intertwine with other biomolecules such as proteins, peptides, nucleic acids, oligonucleotides, and lipids to form glycosylation complex that play important biological functions as well (Cheng et al., 2010). Attributing to their specific structures and unique biological functions, many carbohydrate biomolecules can serve as biomarkers for disease diagnosis and evaluation of medical treatment in patients. To identify or quantify a specific carbohydrate biomarker, mass spectrometry (MS) is one of the most effective technologies applied in drug development and clinical diagnosis. In this chapter, sample preparation and bioanalysis technologies of carbohydrate biomarkers using liquid chromatography (LC) and MS are discussed in the following sections.

#### 24.2 Overview

Carbohydrates can serve as biomarkers for diagnosis of disease and evaluation of treatment of patients. With the advancement of analytical technologies in pharmaceutical industry and clinical laboratory, more and more carbohydrate biomarkers have been identified as disease diagnostic agents.

##### 24.2.1 Clinical Diagnostic Carbohydrate Biomarkers

Aligned with other biomolecules, carbohydrates are considered as one of the most important classes of biomarkers for disease states, protein functions, and developmental states. Based on how the carbohydrate molecules are targeted for the development of therapeutic and diagnostic agents, they can be categorized as the biomarkers for many diseases such as diabetes, mucopolysaccharidosis (MPS) disorders, cancer, infectious diseases, and cardiovascular diseases (CVD). For example, blood glucose (a monosaccharide) value that reflects the oral glucose tolerance along with hemoglobin A<sub>1C</sub> (HbA<sub>1C</sub>) serves as the standard biomarkers for the clinical diagnosis of diabetes (Jeppsson et al., 2002). Other biomarkers derived from sugars—like sugar alcohols (a class of polyols) such as 1,5-anhydro-D-glucitol (1,5-AG) (Katayama et al., 2006), sorbitol (Malone et al., 1980), *myo*-inositol (Tetsuo et al., 1999), and mannitol (Pitkanen, 1996)—have also been demonstrated as biomarkers of diabetes. Besides the monosaccharides and sugar alcohols, oligosaccharides have also been reported as biomarkers. For instance, keratan sulfate (KS) as one of the glycosaminoglycans (GAGs), containing a repeating disaccharide unit with alternating D-galactose and N-acetyl-D-glucosamine (GlcNAc) residues, was identified as a biomarker of mucopolysaccharidosis IVA (MPS IVA, Morquio A syndrome) (Martell et al., 2011; Tomatsu et al., 2010). MPS IVA is an inherited lysosomal storage disease caused by deficiency of N-acetylgalactosamine-6-sulfatase (GALNS), an enzyme required for stepwise degradation of KS (Dvorak-Ewell et al., 2010; Tomatsu et al., 2005). In individuals with MPS IVA, the excess cartilage accumulation of KS disrupts the cartilage, leading to higher levels of KS in the blood and urine, making KS an important biomarker for MPS IVA (Martell et al., 2011; Tomatsu et al., 2010). Another GAG, dermatan

sulfate (DS), accumulates abnormally in several of the MPS disorders (Trowbridge and Gallo, 2002). An excess of DS in the mitral valve is characteristic of myxomatous degeneration of the leaflets, leading to redundancy of valve tissue and, ultimately, mitral valve prolapse (into the left atrium) and insufficiency (Gupta, 2012). Furthermore, recent research for quantitative analysis of certain molecules has led to the observance of the difference of the molecules in quantity in certain diseases, in turn establishing the analyte as a potential novel biomarker. For example, recently quantification of plasma globotriaosylsphingosine (lyso-Gb<sub>3</sub>) and lyso-Gb<sub>3</sub>-related analogs by nano-LC–tandem mass spectrometry (MS/MS) revealed lyso-Gb<sub>3</sub> and its analogs as useful biomarkers for Fabry disease (Sueoka et al., 2015). Most recently, quantitative measurement of glucosylceramide (GluCer) isoform levels by MS/MS analysis in brain tissues at different stages of Parkinson's disease (PD) was utilized to determine if the decreased glucocerebrosidase (GCase) enzyme activity observed in PD tissues is associated with the accumulation of its GluCer substrates in PD brain tissues (Boutin et al., 2016). In cancer research, it was found that many carbohydrates are considered as cancer-associated antigens (CAA). Globo H and Tn antigens, the common CAA, were found on the cell surface of over 90% of solid tumor (Springer, 1995). Another example is sialylated carbohydrates such as sialyl Lewis X (sLeX) that can mediate lung colonization of B16 melanoma cells (Zhang et al., 2002). It is believed that carbohydrates are involved in the pathogenicity of infectious agents. Evidence showed that human influenza virus infection involves sialic acid for binding to hemagglutinin and infection (Liu and Air, 1993). In the cardiovascular area, it is widely recognized that the glycated hemoglobin (GlcHb) level in the blood is a strong indicator of CVD risk in diabetic patients (Dailey, 2007; Gerstein, 2009; Goff et al., 2007; Khaw and Wareham, 2006; Roman and Hancu, 2009; Zhang et al., 2009).

#### **24.2.2 Overview of Bioanalytical Analysis of Carbohydrate Biomarker**

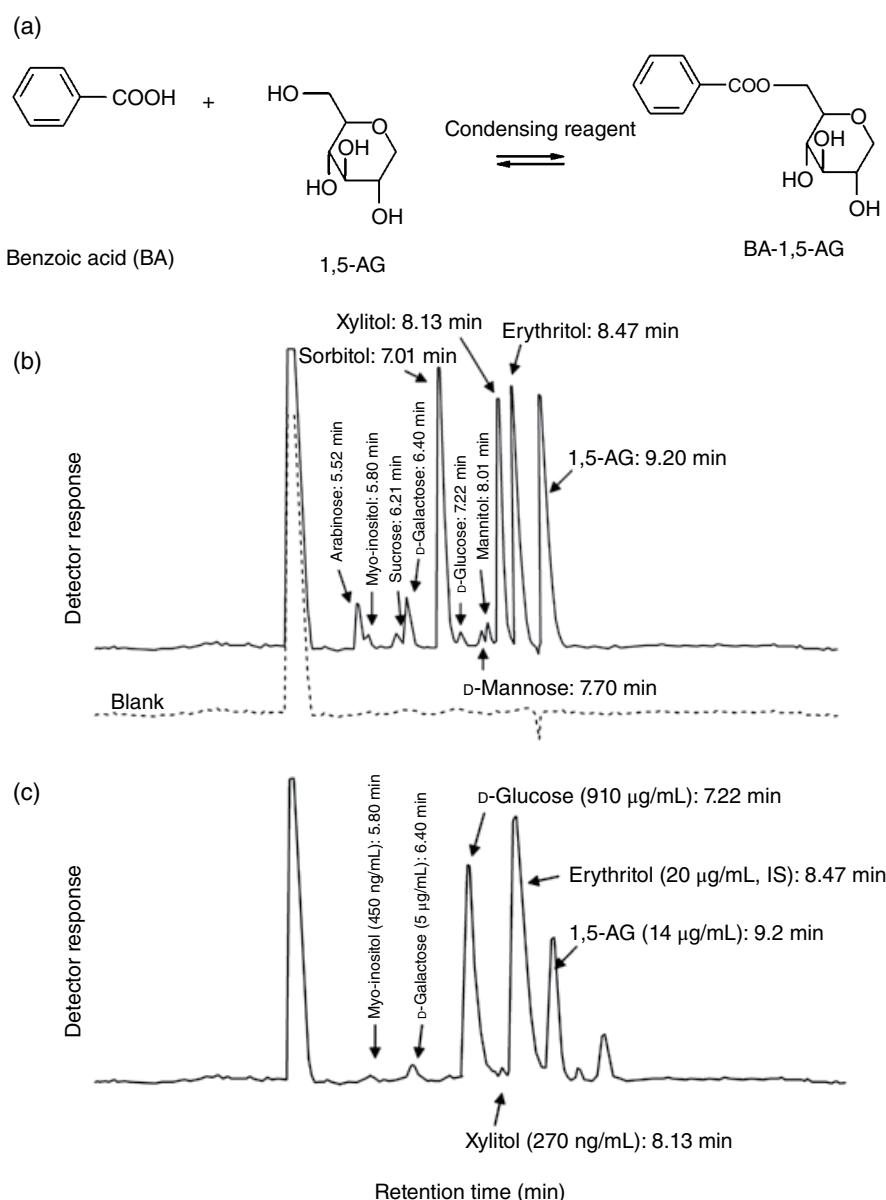
Quantitative bioanalytical support plays a critical role in biomarker analysis. Both enzymatic and chromatographic methods have been used in the determination of plasma or blood sugars. As early as in early 1980s, sorbitol was identified and measured in red blood cells both enzymatically and by gas chromatography (GC) (Malone et al., 1980). Yabuuchi et al. established a simple enzymatic method based on the enzyme pyranose oxidase for determining 1,5-AG in plasma for diagnosis of diabetes (Chusney et al., 1995; Yabuuchi et al., 1989). In addition, enzyme-linked immunosorbent assay (ELISA) was previously reported for analyzing KS using a KS-specific

monoclonal antibody (Staprans and Felts, 1985). KS can also be analyzed by colorimetric assay using dimethyl-methylene blue (DMB) (Farndale et al., 1986; Melrose and Ghosh, 1988). Some other methods measured total GAGs (KS, DS, heparin sulfate (HS), and chondroitin sulfate) by spectrophotometric methods based on the ability of GAGs to bind to DMB (de Jong et al., 1992; Whitley et al., 2002) and Alcian blue (Karlsson et al., 2000). In the 1990s, gas chromatography–mass spectrometry (GC–MS) was a method of choice for carbohydrate analysis, especially for monosaccharides (Pitkanen, 1996; Pitkanen and Kanninen, 1994; Tetsuo et al., 1999). For example, postprandial plasma fructose and glucose concentrations were simultaneously measured by GC–MS in patients with type 2 diabetes who had been admitted to the hospital (Kawasaki et al., 2004). The serum 1,5-AG concentrations (normal range, 78–256 μmol/L) from the same patients were determined by an established enzymatic method using a 1,5-AG clinical test kit (Kawasaki et al., 2004). For GC–MS analysis of carbohydrates, the analytes are usually required to be converted into volatile and stable derivatives. The derivatization process may face several challenges. First, each carbohydrate analyte may have multiple functional groups (e.g., hydroxyls) that could lead to a mixture of derivatization products. Second, each derivatized analyte has different tautomers in solution, thus leading to complex chromatograms due to their retention differences. Third, the chemical bonds in the molecule may not be easy to access for derivatization due to steric hindrance. Fourth, the stability of the analyte after derivatization may need to be taken into consideration for GC–MS analysis. Finally, there is a molecular weight limitation for the analytes that could be analyzed by GC (generally considered not more than 500 Da), while, in a lot of cases, derivation to achieve desirable volatility and thermal stability needs more than two steps of derivatization, which would lead the molecular weight of the derivatized analyte to exceed the limit (500 Da). In later 1990s, high performance liquid chromatography (HPLC) in conjunction with pulsed amperometric detector (PAD) (Chusney et al., 1995; Tanabe et al., 1997), with fluorescence detector (El Rassi et al., 1991; Hase et al., 1978), or with mass spectrometry (HPLC–MS) (Niwa et al., 1994) has been reported for simultaneous determination of sugars or sugar alcohols. It was shown that intra- and inter-assay precisions for the measurement of the human serum 1,5-AG was better with the anion exchange LC-PAD method than the enzymatic method (Chusney et al., 1995). Also, HPLC using a borate form of a strongly anion exchange resin column and an immobilized enzyme reactor for colorimetric detection (a refractive index (RI) detection) was employed to quantify urinary 1,5-AG with the 1.2–300 μmol/L (197–49,248 ng/mL) range (Tanabe et al., 1997).

However, the RI detector did not have sufficient selectivity and sensitivity of detection, and the PAD often requires maintenance for daily analysis (Chusney et al., 1995; Tanabe et al., 1997). In both cases, the reported HPLC methods require a post-column pyranose (Chusney et al., 1995) or glucose-3-dehydroxidase (G3DH) enzymatic reaction (Tanabe et al., 1997) for detection. Later, Katayama et al. developed a simple and rapid HPLC method with pre-column derivatization for simultaneous determination of sugar and sugar alcohols (Figure 24.1) (Katayama et al., 2006). Sugars (*D*-glucose, *D*-galactose,

*D*-mannose, sucrose, and arabinose) and sugar alcohols (xylitol, erythritol, mannitol, sorbitol *myo*-inositol) were derivatized with benzoic acid (BA), followed by HPLC separation and fluorescence detection, with the detection wavelength of  $\lambda_{\text{ex}} 275 \text{ nm}$  and  $\lambda_{\text{em}} 315 \text{ nm}$  on the BA derivatives. The detection limits of sugars were 10–80  $\mu\text{g}/\text{mL}$  and the detection limits of sugar alcohols were 100–1000 ng/mL.

Size exclusion chromatography (SEC) has been used online with electrospray ionization (ESI) MS to separate mixtures of sulfated GAG oligosaccharides (Zaia, 2004;



**Figure 24.1** (a) Scheme of derivatization of 1,5-AG and benzoic acid (BA) reagent. (b) Chromatogram of sugars and sugar alcohols. The sugars and sugar alcohols were derivatized and separated by the LC method with pre-column BA derivatization. (c) Chromatogram of sugars and sugar alcohols in serum by the LC method with pre-column BA derivatization. Source: Katayama et al. (2006). Reproduced with permission of John Wiley & Sons.

Zaia and Costello, 2001). A commercial column (Amersham Biosciences Superdex Peptide) was available to separate di-, tetra-, hexa-, and octasaccharides in a 20 min time period. The method was quite convenient because the oligosaccharide mixture that entered the instrument was well separated, so the complexity of the sample mixture was well resolved. Mobile phase conditions of 0.1 M ammonium acetate and 10% methanol have been found to work for all types of sulfated GAG oligosaccharides. However, SEC coupled with MS may not be suitable for saccharide bioanalytical quantification due to its limited sensitivity.

An ion-pairing reversed-phase chromatographic method has been described for online LC–MS of heparins as well as other acidic carbohydrates (Kuberan et al., 2002; Zaia, 2004). Dibutylamine is used in the mobile phase in the presence of acetic acid where dibutylammonium ions pair with the negatively charged carbohydrates to allow retention on a C18 column, while ion suppression caused by ion pairing would exist, leading to limited sensitivity. A mixture of deprotonated and dibutylammonium-adducted ions was monitored under these conditions for analysis of a heparin pentasaccharide. Significantly, no sulfate losses were observed, which makes this technique suitable for online LC–MS determination of sulfated oligosaccharides. It should be noted that this method is most appropriate when an instrument can be dedicated to heparin-like GAG analysis due to difficulties in removing the ion-pairing reagent. Although it was used for the characterization of oligosaccharides by full mass spectrum acquisition (non-LC–MS/MS), this ion-pairing LC–MS method might have potential utility for saccharide quantification (Kuberan et al., 2002).

Due to the superior specificity and selectivity of MS/MS, targeted LC–MS/MS analysis is an ideal analytical platform for the analysis of carbohydrate molecules in biological fluid. For example, with keratanase II digestion and a reversed-phase graphitized (hypercarb) column, Oguma et al. established and validated a highly sensitive LC–MS/MS method to determine the concentrations of KS-derived disaccharides and their composition ratios from human serum and plasma (Figure 24.2) (Oguma et al., 2007a, 2007b). More recently, urinary concentrations of Gb<sub>3</sub>, lyso-Gb<sub>3</sub>, and related analogs were determined in pediatric and adult Fabry cohorts by LC–MS/MS with hydrophilic interaction chromatography (HILIC) columns (Auray-Blais et al., 2015; Lavoie et al., 2013). Multiplex MS/MS analysis of various plasma lyso-Gb<sub>3</sub>-related analogs was developed by Boutin and Auray-Blais (2014) (Figure 24.3), and Sueoka et al. validated a nano LC–MS/MS method for simultaneous quantification of lyso-Gb<sub>3</sub> and its analogs in the plasma of patients in Fabry disease (Sueoka et al., 2015).

## 24.3 Bioanalytical Method Development for Carbohydrate Biomarkers

### 24.3.1 Sample Preparation

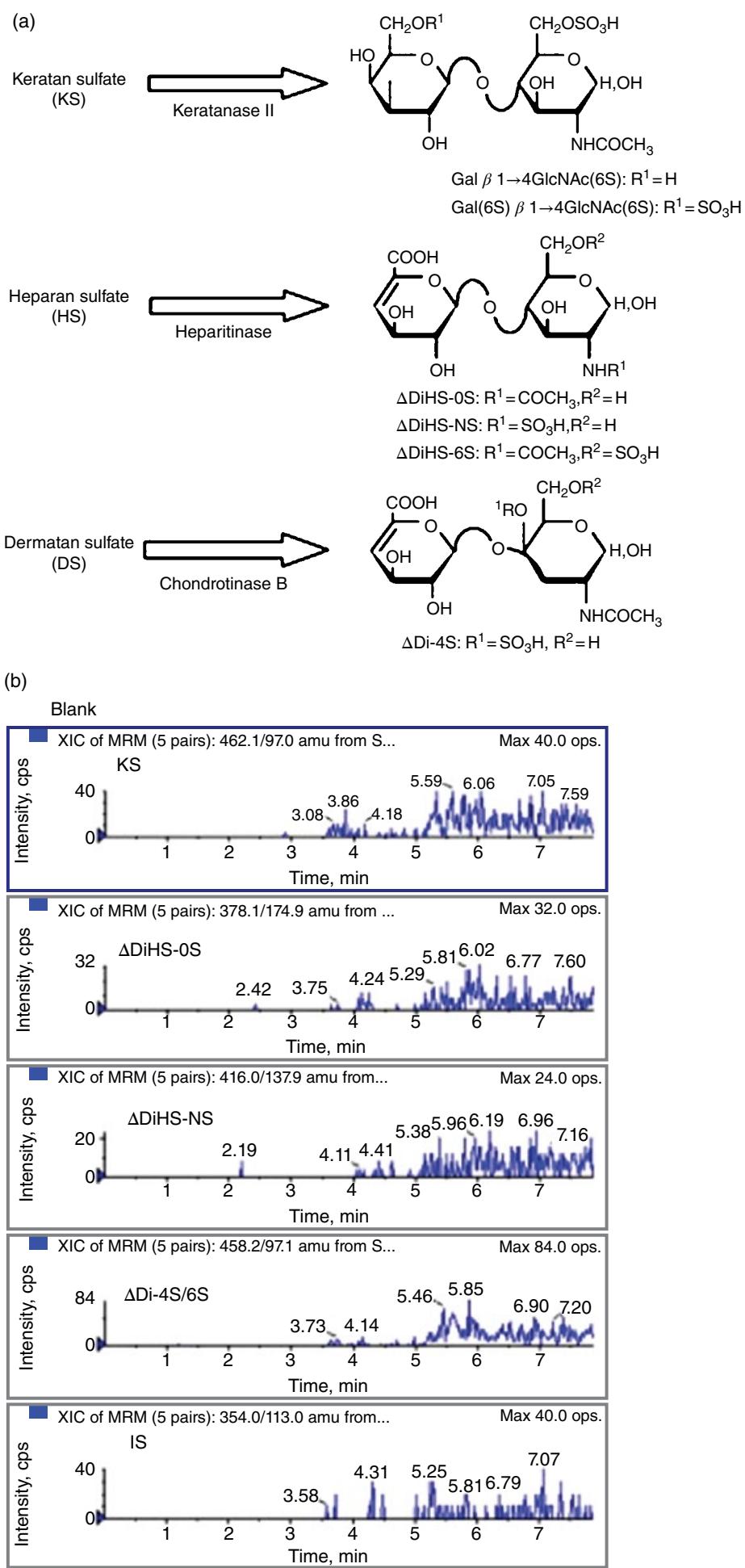
One of the biggest challenges for carbohydrate bioanalytical analysis is to extract the analyte from biological matrices. The sufficient extraction of analytes would allow reduction of the background noise and improvement of the sensitivity in the LC–MS analysis. Depending on the types of carbohydrate analytes, there are a few applicable sample preparation methods, including solid-phase extraction (SPE), liquid–liquid extraction (LLE), derivatization, and enzymatic digestion/chemical reduction. These commonly used sample preparation methods should be evaluated and developed based on the characteristics of the carbohydrate molecules.

#### 24.3.1.1 Sample Preparation by Solid-Phase Extraction (SPE)

In the study conducted by Boutin and Auray-Blais in 2014, a very detailed SPE sample preparation method was reported for the quantitative LC–MS/MS analysis of lyso-Gb<sub>3</sub> and its related analogs in human plasma (Figure 24.3). Other studies used very similar SPE sample preparation method (Auray-Blais et al., 2015; Lavoie et al., 2013; Sueoka et al., 2015). SPE procedures using mixed-mode cation exchange cartridges demonstrated a high selectivity for basic compounds and thus help significantly reduce potential interferences from the matrix, including neutral and acidic compounds as well as salts (Lavoie et al., 2013). Briefly, aliquots of well-mixed plasma from Fabry patients or healthy controls were first acidified with H<sub>3</sub>PO<sub>4</sub> (2% in water) followed by the addition of lyso-Gb<sub>3</sub>-Gly as internal standard (IS). Sample mixtures were then gone through mixed-mode cation exchange cartridges, followed by wash with 2% formic acid in water and 0.2% formic acid in methanol. The analytes lyso-Gb<sub>3</sub>, with its related analogs, and lyso-Gb<sub>3</sub>-Gly (IS) were eluted with 2% ammonia in methanol. The eluates were dried under nitrogen and resuspended in 50% acetonitrile/0.1% formic acid/water for ultrahigh performance liquid chromatography (UPLC)–MS/MS analysis.

Besides being applied to plasma samples, SPE was also utilized to extract the carbohydrate biomarkers from tissue samples, such as GluCer isoforms from brain tissues. In the study of Boutin et al. (2016), homogenization was first performed for the extraction of GluCer and galactosylyceramide (GalCer) isoforms from frozen human or murine brain tissue specimen. Tissue samples were homogenized using zirconium oxide beads in methanol, which was used as the solvent in order to denature

**Figure 24.2** Enzymatic digestion of GAGs to each disaccharide (a). Representative MRM chromatograms of extracts obtained from control serum without enzymatic digestion (b) and calibration standard sample of lower limit of quantitation (LLOQ) (c). Source: Oguma et al. (2007a, 2007b). Reproduced with permission of Elsevier.



(c)

LLOQ

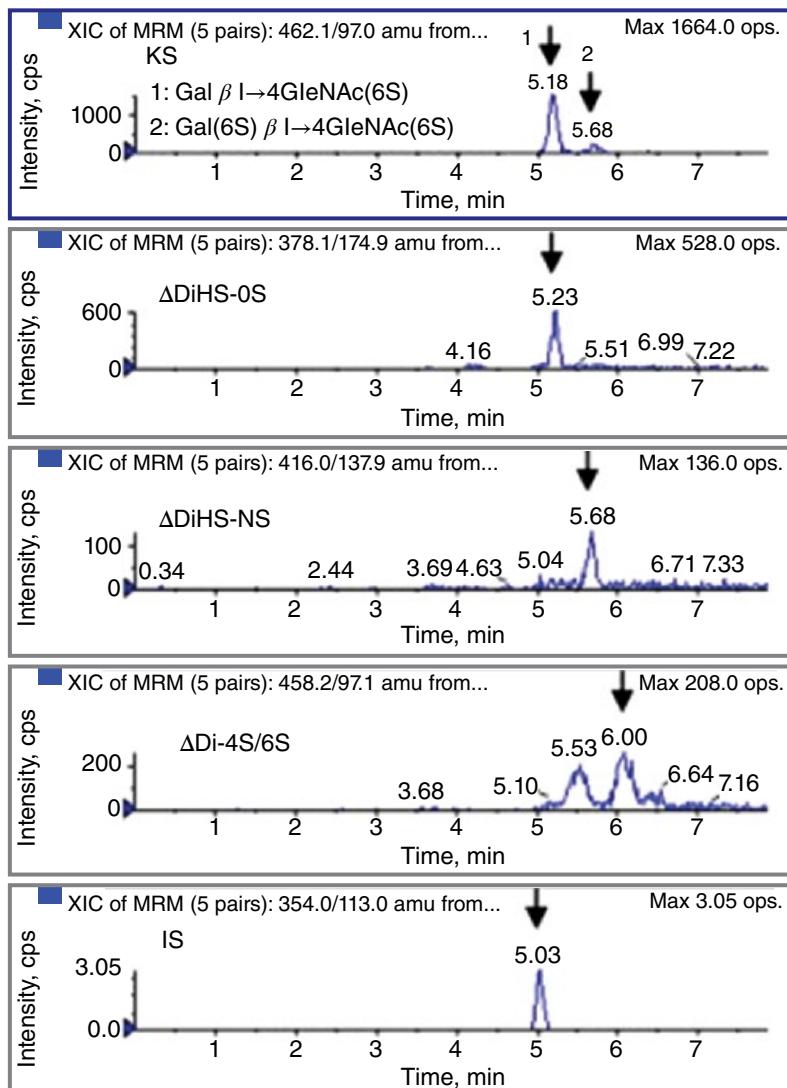


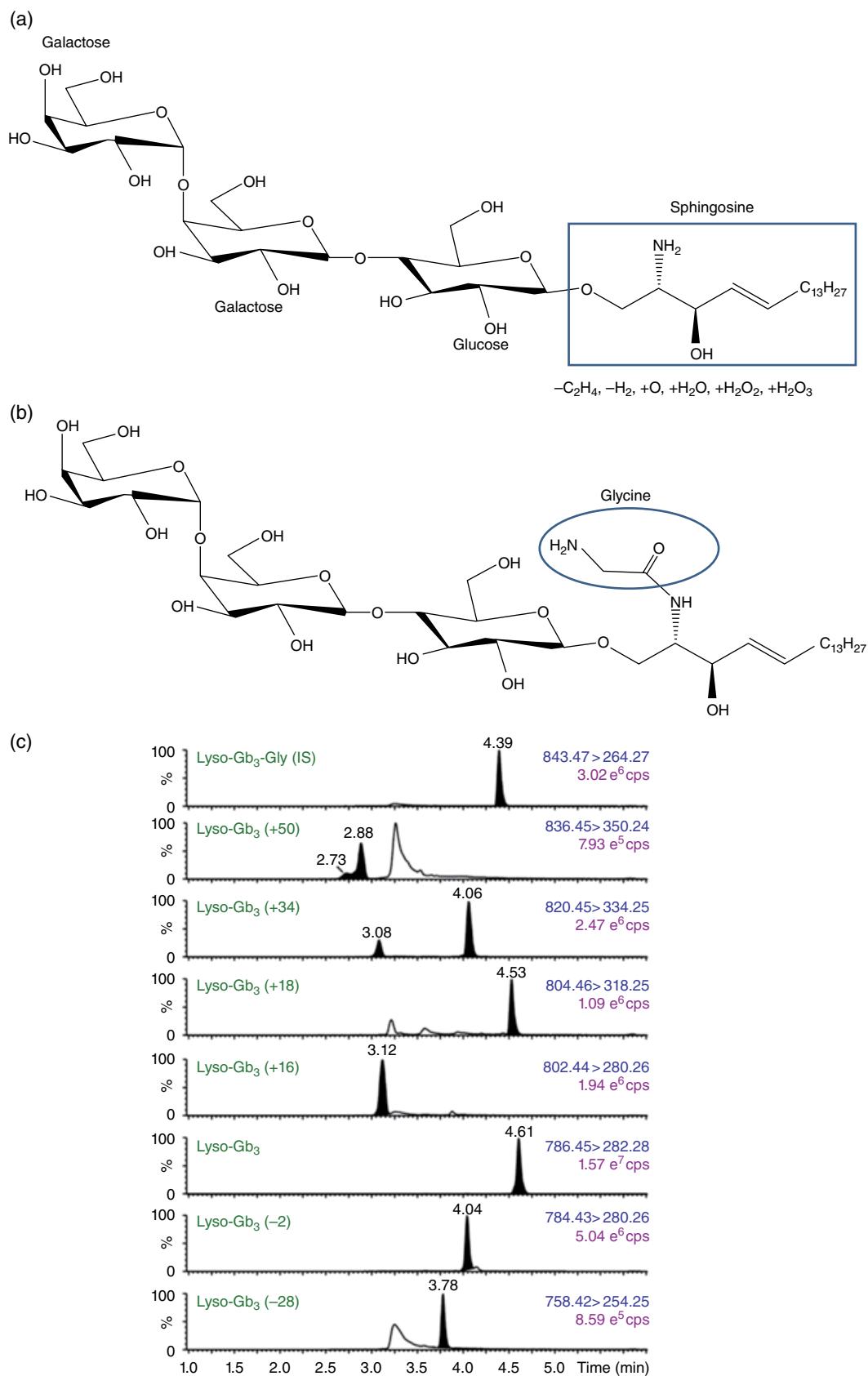
Figure 24.2 (Continued)

proteins and prevent enzymatic reactions. The brain tissue homogenates were then added with the IS (GluCer(C16:0)-d<sub>3</sub>) in DMSO and mixed with acetone and water, followed by vortexing and centrifuging to retrieve the supernatant. The supernatants were diluted with water and loaded on a hydrophilic-lipophilic balance (HLB) SPE cartridge. The cartridge was washed with 60% methanol/30% water/10% acetone, and the samples were eluted with 90% acetone/10% methanol. Finally, the sample was evaporated to dryness under nitrogen and resuspended with DMSO: mobile phase A (1:4, v:v) (phase A: 95% acetonitrile/2.5% methanol/2.5% water/0.5% formic acid/5 mM ammonium formate) for LC-MS/MS analysis.

#### 24.3.1.2 Sample Preparation by Liquid-Liquid Extraction (LLE)

In the study reported by Gold et al. (2013), an LLE sample preparation method was reported for the quantitative LC-MS/MS analysis of lyso-Gb<sub>3</sub> and lyso-ene-Gb<sub>3</sub> from human plasma and urine.

Lyso-Gb<sub>3</sub> was extracted from plasma by a modification of the method published by Bligh and Dyer (1959). Plasma sample was first spiked with the IS ([5,6,7,8,9] <sup>13</sup>C<sub>5</sub>-labeled lyso-Gb<sub>3</sub> in methanol) followed by the addition of relatively large volume of methanol and chloroform. The sample mixture needed to be stirred well, left for 30 min at room temperature and then centrifuged. The centrifugation would allow the removal of precipitated



**Figure 24.3** (a) Chemical structure of globotriaosylsphingosine (lyso-Gb<sub>3</sub>), showing six modifications of sphingosine chain observed in plasma. (b) Structure of lyso-Gb<sub>3</sub>-Gly used as the internal standard (IS). (c) MRM chromatograms of lyso-Gb<sub>3</sub> and its six related analogs and lyso-Gb<sub>3</sub>-Gly, used as the IS, in a plasma sample from an untreated Fabry male. Source: Boutin and Auray-Blais (2014). Reproduced with permission of American Chemical Society.

protein, and the supernatants were then extracted with chloroform. The samples would be separated to two phases by the LLE procedure. The upper phase (aqueous phase) containing the analyte was transferred, and the lower phase (chloroform phase) was extracted again by methanol and water, from which the upper phase was taken and pooled with the previous upper phase extract. The sample was then dried under nitrogen and redissolved in methanol. The recovery of lyso-Gb<sub>3</sub> with this method was reported as high as 98% (Gold et al., 2013). Calibration curves were constructed with control plasma and urine supplemented with lyso-Gb<sub>3</sub>. In addition to lyso-Gb<sub>3</sub>, lyso-ene-Gb<sub>3</sub> was quantified.

Similar to the extraction from plasma, lyso-Gb<sub>3</sub> and lyso-ene-Gb<sub>3</sub> were extracted from urine also with a modification of the method of Bligh and Dyer (van Breemen et al., 2011). The urine samples spiked with IS ([5,6,7,8,9]<sup>13</sup>C<sub>5</sub>-labeled lyso-Gb<sub>3</sub>) were extracted with methanol and chloroform in the upper phase, which was then evaporated to dryness and dissolved in *n*-butanol saturated with water. The dissolved sampler was extracted again with water saturated with butanol. The resulting upper (butanol) phase was transferred, evaporated to dryness, and reconstituted in methanol for LC-MS/MS analysis. The second sample cleanup step by butanol extraction was necessary to obtain sufficiently clean analyte preparations. Lyso-Gb<sub>3</sub> was nearly quantitatively recovered in the butanol phase, and the overall recovery of lyso-Gb<sub>3</sub> with this method was 80% (Gold et al., 2013).

#### 24.3.1.3 Sample Preparation by Derivatization

Carbohydrate or sugar molecules are often very polar and highly aqueous soluble and require derivatization or HILIC for LC-MS analysis. Derivatization with polar moieties could help retain the molecules on the conventional reversed-phase stationary phase LC column. The derivatization of sugar and sugar alcohols was reported by Katayama et al. (2006) for a quantitative LC analysis with fluorescence detection. The sugar and sugar alcohol analytes were first extracted from serum followed by derivatization with BA (Figure 24.1a). BA was selected as the labeling reagent, because a smaller reagent molecule was better for separation of the sugar derivatives. The detection limits of 10–80 µg/mL for sugars and 100–1000 ng/mL for sugar alcohols were obtained by fluorescence detection ( $\lambda_{\text{ex}} 275 \text{ nm}$  and  $\lambda_{\text{em}} 315 \text{ nm}$ ). Each reagent concentration was studied and set as follows: BA reagent solution, 0.02% w/v; 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate (IDC) solution, 2.0% w/v; and 4-piperidinopyridine, 0.01% w/v. D-Glucose, arabinose, D-galactose, D-mannose, and sucrose were reacted over 80°C, but fucose, fructose, lactose, rhamnose, and ascorbic acid gave no peaks in the chromatogram (not reacted with the derivatization

reagent). 1,5-AG and other sugar alcohols (xylitol, erythritol, mannitol, sorbitol *myo*-inositol) were also derivatized with BA at 80°C for 60 min, from which the highest detector response was obtained. The derivatization products from phenols and alcohols were determined as esters (Katayama and Taniguchi, 1993; Katayama et al., 1993, 2001). The BA derivatives of sugar and sugar alcohol were suspected to be the monosubstituted products previously reported for alkyl alcohol (Katayama et al., 1991) and bisphenol A (Katayama et al., 2001). It was also suspected that fucose, fructose, lactose, rhamnose, ascorbic acid, mannitol, and *myo*-inositol were not derivatized by BA owing to steric hindrance. Although sugars and sugar alcohols were quantified by LC analysis coupled with fluorescence detection, this sample preparation method could potentially be applied for LC-MS analysis, since the derivatization of carbohydrates could significantly decrease the polarity of these sugars and help retain them on the conventional reversed-phase stationary phase using a mobile phase with higher organic content, which facilitates more efficient ionization of these analytes.

The following example demonstrates a sample preparation method for quantification of GAG metabolites (sulfated *N*-acetylhexosamine-containing mono- and disaccharides) from urine or plasma from patients with derivatization using 1-phenyl-3-methyl-pyrazolone (PMP) and SPE (Ramsay et al., 2003; Zaia, 2009). Samples of plasma (20 µL) and urine (1.0 µmol creatinine equivalents) were lyophilized prior to derivatization, and the lyophilized samples of urine or plasma were derivatized with PMP, from which excess reagent was removed by chloroform extraction and the aqueous layer was loaded to a C18 SPE cartridge. The cartridge was washed with chloroform and analytes of interest were eluted out with 50% acetonitrile. PMP-derivatized sulfated mono- and disaccharides were then analyzed using negative ion ESI-MS/MS. This method has been used to quantify mono- and disulfated HexNAc and monosulfated HexNAc-HexA in plasma of mucopolysaccharidosis patients (Ramsay et al., 2003). The method has also been used to monitor the levels of *N*-acetylhexosamine and *N*-acetylhexosamine/uronic acid as biomarkers in animal models with the animals treated with different doses of drugs in an enzyme replacement therapy for mucopolysaccharidosis type VI disease (Crawley et al., 2004).

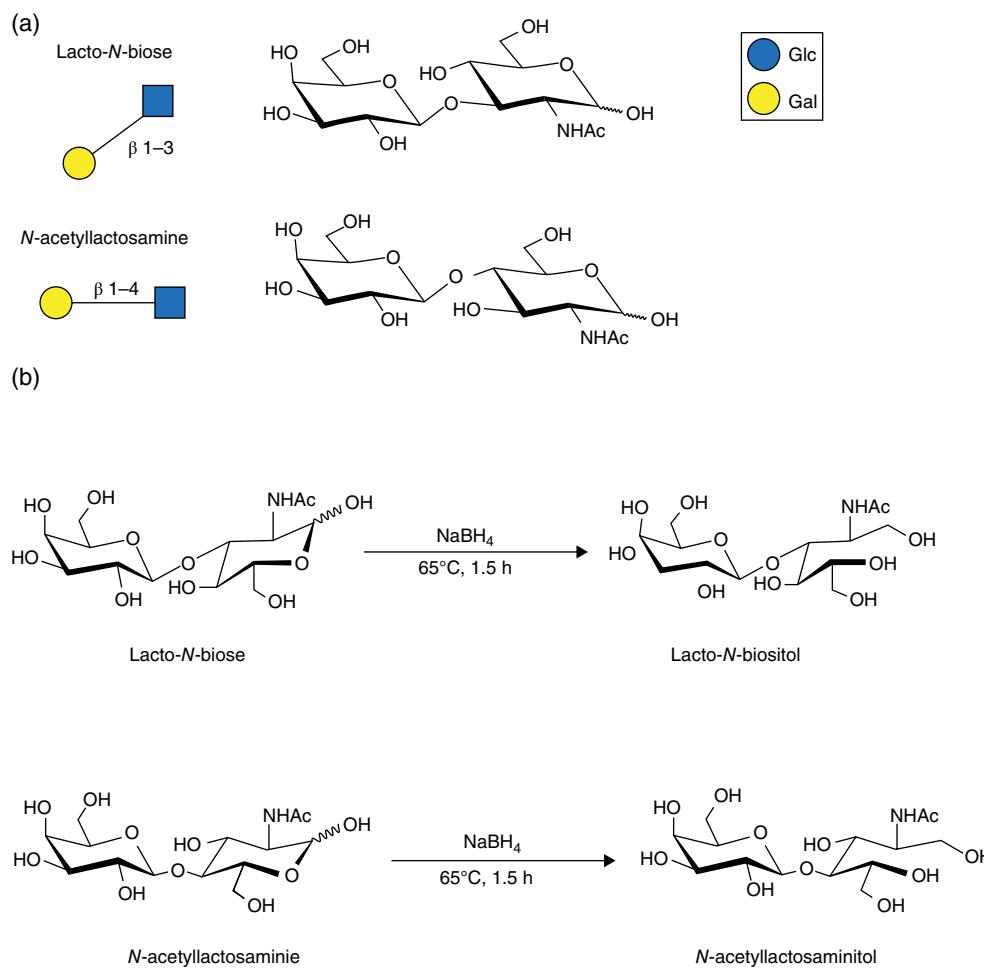
#### 24.3.1.4 Sample Preparation by Enzymatic Digestion or Chemical Reduction

For the analysis of disaccharides derived from KS, HS, and DS, enzymatic digestion by keratanase II, heparitinase, and chondroitinase B followed by centrifugation and filtering was reported as major step for sample

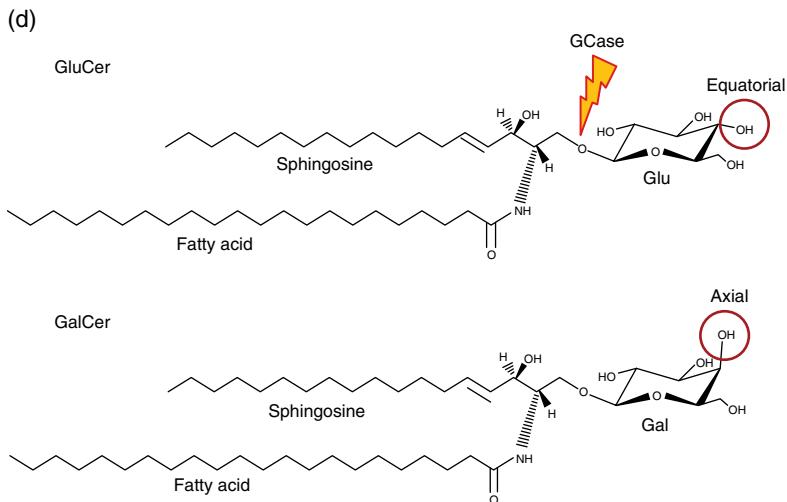
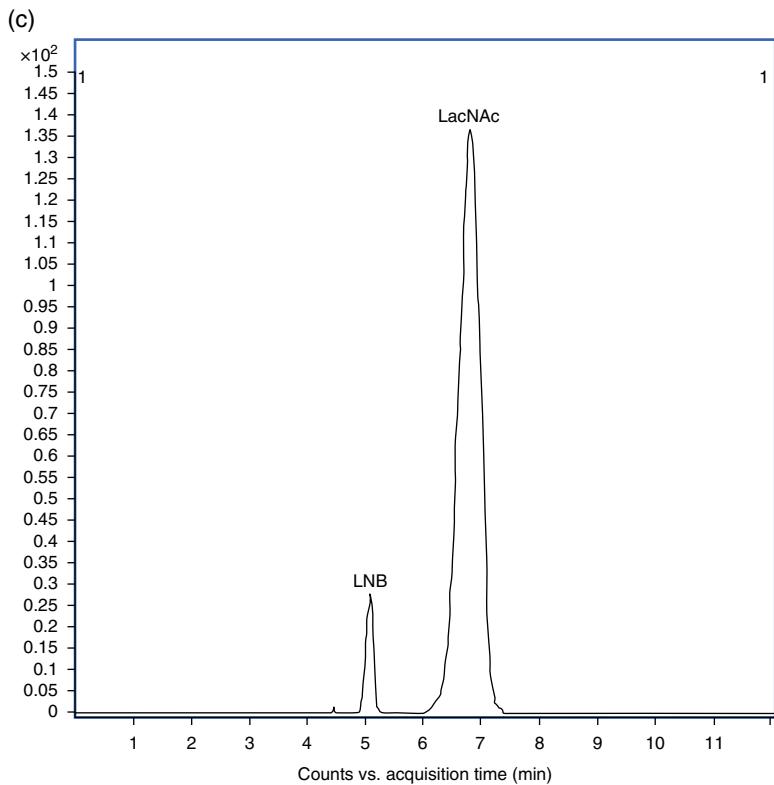
preparation (Martell et al., 2011; Oguma et al., 2007a, 2007b). Serum or plasma samples were first spiked with the IS (e.g., chondrosine) and incubated with the keratanase II, heparitinase, and chondroitinase B mixture solution in tris-hydrochloric acid buffer. The samples were then centrifuged and filtered for LC-MS injection. Calibration samples for KS, HS, and DS disaccharides were prepared by spiking the serum or plasma with the analyte and IS, followed by the same enzymatic digestion process (Oguma et al., 2007a).

In the study of Balogh et al. (2015), a quantitative LC-MS/MS analytical method for *N*-acetyllactosamine (LacNAc) and lacto-*N*-biose (LNB) (Figure 24.4), two

major building blocks of oligosaccharides in human milk, was developed and validated. The sample preparation procedure was more complicated, involving both LLE and SPE cleanup and analyte reduction (Balogh et al., 2015). The fat and protein removal procedure was adapted from Ninonuevo et al. with small modifications (Ninonuevo et al., 2006). The milk samples (10 mL) were subjected to LLE with four volumes of a chloroform-methanol (2:1, v/v) mixture. After vortexing and centrifuging, the upper phase was then collected, and the lower chloroform phase and the denatured protein were discarded. The whole procedure was repeated with the upper phase to ensure the removal of all fat and protein from the sample. The upper



**Figure 24.4** (a) The schematic structures of the lacto-*N*-biose and the *N*-acetyllactosamine along with the structure of the disaccharides are also shown. (b) The mechanism of the reduction with NaBH<sub>4</sub> at 65°C for 1.5 h in the case of both disaccharides. (c) The chromatogram obtained from a human milk sample from the first day of lactation. Source: Balogh et al. (2015). Reproduced with permission of Elsevier. (d) Structures of glucosylceramide (GluCer) and galactosylceramide (GalCer). Both molecules (GluCer and GalCer) are comprised of a sugar molecule conjugated with a sphingosine molecule and a fatty acid chain. According to different lengths of fatty acid chains, it would correspond to different GluCer/GalCer couples. The cleavage site of the glucocerebrosidase (GCase) enzyme is indicated on the GluCer molecules. The circled structures indicate the hydroxyl group differentiating GluCer and GalCer by its axial or equatorial configurations. Glu (glucose), Gal (galactose). Source: Boutin et al. (2016). Reproduced with permission of American Chemical Society.



**Figure 24.4** (Continued)

layer collected after the second extraction and centrifugation was dried under nitrogen and lyophilized. NaBH<sub>4</sub> solution was then used to reduce LacNAc/LNB to their alditol form (Figure 24.4c). After the reduction step, a nonporous graphitic carbon SPE cartridge was used to remove borate salts. Each cartridge was preconditioned with methanol and water, and after loading the sample, each cartridge was washed with deionized water at low temperature (2–3°C) to remove borate salts. For the

elution of the reduced LacNAc/LNB, acetonitrile/water (1:1, v/v) was used (Arias et al., 2003). The resulting sample was lyophilized and analyzed further with LC-MS.

#### 24.3.2 Chromatography and Column Options

Carbohydrate or sugar molecules are often very polar with high aqueous solubility and often have to be applied to HILIC or chromatography with graphitized carbon

columns for LC-MS analysis. In some cases, reversed-phase chromatography is also used for carbohydrate analysis. Table 24.1 summarizes the analytical columns reported for different carbohydrate analyses.

#### 24.3.2.1 HILIC for LC-MS/MS Bioanalysis

Historically normal-phase liquid chromatography (NP-LC) has been widely used to separate various compounds, from nonpolar to highly polar compounds including sugar molecules (Buszewski and Noga, 2012). HILIC is an alternative HPLC mode for separating polar compounds. Like NP-LC, HILIC employs traditional polar stationary phases such as silica, amino, or cyano (Garbis et al., 2001; Guo and Gaiki, 2005; Li and Huang, 2004; Olsen, 2001), but the mobile phase used is similar to those employed in the reversed-phase LC mode. HILIC also allows the analysis of charged substances, as in ion chromatography (IC). HILIC has been widely applied to the analysis of small polar molecules, including nucleosides, nucleotides/oligonucleotides, amino acids, peptides and proteins, saccharides, glycosides, oligosaccharides, hydrophilic drugs, and other small polar or ionizable compounds (Buszewski and Noga, 2012). LC-MS/MS analysis of lyso-Gb<sub>3</sub> or lyso-Gb<sub>3</sub> analogs using Atlantis HILIC column containing silica stationary phase was reported (Auray-Blais et al., 2015; Lavoie et al., 2013). Lyso-Gb<sub>3</sub> and its seven analogs extracted from urine samples were well retained and resolved on the HILIC column. HILIC was chosen over reversed-phase LC because this strategy provided a better alignment of

the retention times of lyso-Gb<sub>3</sub> and its analogs with that of lyso-Gb<sub>3</sub>-Gly IS and a better correction of the MS response (Lavoie et al., 2013).

In the study by Boutin et al., HALO HILIC column was used for the LC-MS/MS analysis of GluCer isoforms. The separation of GluCer and isobaric GalCer isoforms was achieved under an isocratic condition using mobile phase consisting of acetonitrile, methanol, and water containing formic acid and ammonium formate (Boutin et al., 2016).

In the study by Katayama and colleagues, C4, C8, ODS (octadecyl or C18), and amino- (NH<sub>2</sub>-) and amide-type columns have been tried for the separation of sugar and sugar alcohols by LC analysis with fluorescence detector (Katayama et al., 2006). A TSK Amide-80 column gave optimal separation of BA-sugar alcohol derivatives, and 11 analytes were simultaneously separated and determined with a 10 min isocratic gradient (Figure 24.1b and c).

#### 24.3.2.2 Porous Graphic Hypercarb Chromatography for LC-MS/MS Bioanalysis

The porous graphitic carbon (PGC) material has the ability to retain very polar compounds and also has other unique properties as a stationary phase for LC analysis (Ali et al., 2013; Melmer et al., 2011; Ross and Knox, 1997). Its chemical surface properties distinguish it from more conventional LC column packings such as bonded silica gels and polymers. PGC particles are spherical and fully porous with a porosity of approximately 75% (Ali et al., 2013). The surface of

**Table 24.1** Carbohydrates analyzed with different analytical columns reported in literature.

Carbohydrate analyte	Analytical column	References
Lyso-Gb <sub>3</sub> and analogs	Atlantis HILIC silica (2.1 × 50 mm, 3 μm, Waters) HALO HILIC 2.7 (4.6 × 150 mm, 2.7 μm, Advanced Materials Technology) Reversed-Phase ACQUITY UPLC BEH C18 (2.1 × 50 mm, 1.7 μm, Waters) ZORBAX 300SB-C18 nano column (0.1 × 150 mm, 3 μm, Agilent)	Auray-Blais et al. (2012, 2015), Lavoie et al. (2013), Gold et al. (2013), Boutin and Auray-Blais (2014), Boutin et al. 2016, Sueoka et al. (2015)
Disaccharides Galβ1 → 4GlcNAc (6S) and Gal (6S) β1 → 4GlcNAc(6S) derived from keratan, heparan, and dermatan sulfates; lacto- <i>N</i> -biose, <i>N</i> -acetyllactosamine	C18 polymeric silica column (300 μm × 250 mm, Vydac) Hypercarb (2.0 × 50 mm, or 2.0 × 100 mm, or 2.0 × 150 mm, 5 μm, Thermo Scientific)	Kuberan et al. (2002), Oguma et al. (2007a, 2007b), Martell et al. (2011), Balogh et al. (2015)
Sugars (D-glucose, D-galactose, D-mannose, sucrose, arabinose); sugar alcohols (xylitol, erythritol, mannitol, sorbitol, myo-inositol)	TSK Amide-80 column (4.6 × 250 mm, 5 μm particle size, Tosoh)	Katayama et al. (2006)

PGC is crystalline and highly consistent with no micropores (Ali et al., 2013). At the molecular level, PGC is made up of sheets of hexagonally arranged carbon atoms linked by the same conjugated 1,5-order bonds, which are present in any large polynuclear aromatic hydrocarbon (Ross and Knox, 1997). PGC behaves similarly to a strongly retentive alkyl-bonded silica gel for nonpolar analytes. The retention of polar compounds can be explained by the polar retention effect on graphite (PREG) whereby analytes of increasing polarity showed a high affinity toward the graphite surface (Ross and Knox, 1997). With conventional alkyl-bonded silica, the addition of a polar group to a molecule will normally reduce retention in the reversed-phase mode, whereas with PGC retention is reduced to a much smaller extent or may even increase. Tanaka and coworkers (1991) plotted  $\log k$  for the various stationary phases against  $\log P$  (where  $P$  is the octanol–water partition ratio) and showed that the retention of polar compounds on PGC was much higher than expected, exhibiting  $k$  values 4–15 times higher than expected on the basis of their  $\log P$ . This behavior makes PGC well suited to the separation of very polar and ionized solutes such as carbohydrates and compounds with several hydroxyl, carboxyl, amino, and other polar groups (Antonio et al., 2007; Bieri et al., 2006; Gaudin et al., 2007; Vial et al., 2001; West et al., 2010).

LC-MS/MS analysis of disaccharides prepared from sulfated N-linked oligosaccharides by using PGC columns was reported (Martell et al., 2011; Oguma et al., 2007a, 2007b). An LC-MS/MS method using a hypercarb column was developed and validated for the analysis of KS-derived disaccharides Gal $\beta$ 1–4GlcNAc(6S) and Gal(6S) $\beta$ 1–4GlcNAc(6S) in human urine and plasma after keratanase II digestion. Mobile phase contains ammonium acetate and tributylamine (Martell et al., 2011). Other studies were reported to separate and quantify disaccharides prepared from KS, HS, and DS by LC-MS/MS using a longer hypercarb column with a gradient elution of acetonitrile–0.01 M ammonium bicarbonate (pH 10) (Oguma et al., 2007a, 2007b). In another study, a hypercarb column was also used in the LC-MS/MS analysis for the identification and quantification of two major building blocks of human milk oligosaccharides, LNB and LacNAc (Balogh et al., 2015). The main advantage of this method is the capability of retaining and separating these highly related linkage isomers with only subtle chromatographic differences (Figure 24.4). With this method, identification of both unbound LNB and LacNAc and detection of their concentration changes throughout the first week of lactation were achieved (Balogh et al., 2015).

#### 24.3.2.3 Reversed-Phase Chromatography for LC-MS/MS Bioanalysis

LC-MS/MS analysis of carbohydrate biomarkers by using reversed-phase LC C18 columns was also reported (Boutin and Auray-Blais, 2014; Gold et al., 2013; Sueoka et al., 2015). Different from previous studies using HILIC columns in the bioanalysis of lyso-Gb<sub>3</sub> and its analogs, Boutin et al. and Gold et al. utilized the reversed-phase C18 column to quantify lyso-Gb<sub>3</sub> and its analogs from human plasma and urine. Lyso-Gb<sub>3</sub> and its analogs contain the 15-carbon sphingosine chain, which is relatively more hydrophobic than regular sugar molecules. This feature in the structures of lyso-Gb<sub>3</sub> and its analogs makes them well suited for the retention with a reversed-phase C18 column.

Sueoka et al. employed a ZORBAX 300SB-C18 nano-flow column for simultaneous determination of lyso-Gb<sub>3</sub> and its analogs in human plasma by LC-MS/MS (Sueoka et al., 2015). Superior sensitivity was achieved in this nano-LC-MS/MS assay using nano-flow rate (0.5  $\mu$ L/min). The hydrophilicity of the oligosaccharides limits the surface activity in ESI droplets in regular flow LC. With small electrospray droplets under nano-flow condition, the sensitivity is significantly enhanced (Zaia, 2004). The limit of detection (LOD) of lyso-Gb<sub>3</sub> with the assay method is 0.01 nM, which is lower than that (2–10 nM) for conventional HPLC method (Aerts et al., 2008; Mitobe et al., 2012; Rombach et al., 2010; Togawa et al., 2010; van Breemen et al., 2011) and that (0.05 nM) for the most sensitive assay method involving MS established previously (Gold et al., 2013).

#### 24.3.2.4 Reversed-Phase Ion-Pair Chromatography for LC-MS Bioanalysis

LC-MS analysis of HS oligosaccharides using C18 polymeric silica column with ion-pairing reagent was reported (Kuberan et al., 2002). The HPLC separations were performed at the flow rate of 5  $\mu$ L/min with mobile phase containing acetic acid and ion-pairing reagent (e.g., dibutylamine, tributylamine, or triethylamine). In the ion-pairing reversed-phase chromatography, when the compound is very polar, the analyte could bind to the ion-pairing reagent, which is adsorbed in the stationary phase, retaining the compound on the column. The ion-pairing reagent has an ionic end and a nonpolar tail, such as in dibutylamine. The reagent is added to the mobile phase and allowed to come to equilibrium with the column, on which the nonpolar end of the reagent is held strongly by the nonpolar stationary phase (e.g., C8 or C18), leaving the charged functional group sticking out into the mobile phase. Then the ionic species of the opposite charge (e.g., the analyte) can be attracted to the immobilized ion-pairing reagent, providing chromatographic retention. HS is a highly acidic polysaccharide

with repeating disaccharide units consisting of a glucosamine and hexuronic acid (ido- and/or gluco-) (Kuberan et al., 2002), and the HS disaccharide units could bind to the basic ion-pairing reagent dibutylamine to form the hydrophobic complex retaining on the column, and with increasing level of the organic mobile phase, the analyte would be eluted off the column. Non-sulfated, single sulfated disaccharide, double sulfated disaccharide, triple disaccharide, and pentasaccharides were reported being eluted with increasing organic content in the mobile phase (Kuberan et al., 2002). This reversed-phase ion-pairing capillary LC-MS method was able to analyze sizes up to tetracontasaccharide with high resolution in a single run and was amenable to negative ion electrospray MS in which sodium adduction and fragmentation were avoided. One disadvantage is that ion-pairing reagents generally have high boiling points (high column temperature), which may cause ion suppression issues, leading to the sensitivity limit. Another disadvantage is the long retention time of the analyte on column resulting in long chromatographic time.

#### 24.3.3 LC-MS/MS Analysis

Electrospray ionization tandem mass spectrometry (ESI-MS/MS) has become a common tool for small molecule quantification for several decades. For carbohydrate biomarkers, ESI can be operated with reversed-phase ion-pairing, normal-phase, PGC chromatography, or SEC using compatible mobile phases. Both ESI positive and negative ionization modes have been employed for LC-MS/MS analysis.

Multiple reaction monitoring (MRM) acquisition in positive ionization mode coupled with graphitized carbon chromatography was adapted in the study of Balogh et al. (2015) for quantifying LacNAc and LNB, which are regioisomers. LacNAc and LNB are the two major building blocks of human milk oligosaccharides in human milk (Figure 24.4a–c). The fragmentation of the precursor ion of *m/z* 408—the sodium adduct of each reduced sugar,  $[M+Na]^+$ —resulting in the fragment ion of *m/z* 246 was chosen as quantifier ion for LacNAc and LNB (Balogh et al., 2015).

Both MRM and Q-TOF in positive ionization mode were used by Boutin to analyze novel plasma lyso-Gb<sub>3</sub>-related analogs in Fabry disease (Boutin and Auray-Blais, 2014). The metabolomic study previously performed on plasma samples of Fabry patients by TOF-MS revealed four new lyso-Gb<sub>3</sub> analogs [lyso-Gb<sub>3</sub>(-2, -H<sub>2</sub>), (+16, +O), (+18, +H<sub>2</sub>O), and (+34, +H<sub>2</sub>O<sub>2</sub>)] as biomarkers characteristic of Fabry disease (Boutin and Auray-Blais, 2014; Dupont et al., 2013). The MRM methods performed on triple-quadrupole mass spectrometers are much more sensitive than the TOF-MS full-scan

methods used to discover new biomarkers. For this reason, the presence of the biomarkers previously detected in urine, but not in plasma using TOF-MS method, was verified in plasma using MRM (Figure 24.3c) (Boutin and Auray-Blais, 2014). The lyso-Gb<sub>3</sub>(-28) and (+50) analogs were detected as novel biomarkers in plasma of Fabry patients. Figure 24.3 presents MRM chromatograms and transitions for lyso-Gb<sub>3</sub> and its six related analogs and the lyso-Gb<sub>3</sub>-Gly IS in the plasma of an untreated Fabry male. For each MRM transition, the fragment ion reflecting the highest sensitivity was selected. In the case of lyso-Gb<sub>3</sub> and lyso-Gb<sub>3</sub>(-28, -C<sub>2</sub>H<sub>2</sub>) and (-2, -H<sub>2</sub>) analogs, the fragment ion corresponds to the respective sphingosine moiety with the loss of a water molecule (-H<sub>2</sub>O). For lyso-Gb<sub>3</sub> (+16, +O) and the lyso-Gb<sub>3</sub>-Gly as IS, it corresponds to the sphingosine moiety with the loss of two water molecules (-2H<sub>2</sub>O). Finally, for the lyso-Gb<sub>3</sub> (+18, +H<sub>2</sub>O), (+34, +H<sub>2</sub>O<sub>2</sub>), and (+50, +H<sub>2</sub>O<sub>3</sub>) analogs, it corresponds to the respective sphingosine moiety. In the case of the lyso-Gb<sub>3</sub> (+34, +H<sub>2</sub>O<sub>2</sub>) analog, two major peaks, separated by approximately 1 min, were observed on its MRM chromatogram (the third channel in Figure 24.3c). When using Q-TOF MS to study the fragmentation of the two peaks of lyso-Gb<sub>3</sub> (+34, +H<sub>2</sub>O<sub>2</sub>), it was demonstrated that the fragmentation spectra from the two peaks are similar, indicating that the two peaks correspond to structural isomers of lyso-Gb<sub>3</sub> (+34, +H<sub>2</sub>O<sub>2</sub>) (Boutin and Auray-Blais, 2014). For the quantitative analysis, these two peaks were integrated together and their areas were summed. The peak corresponding to lyso-Gb<sub>3</sub> (+50, +H<sub>2</sub>O<sub>3</sub>) presents a peak splitting (the second channel in Figure 24.3c), which was attributed to different structural isomers generated when hydroxyl groups are added to the sphingosine chain to give rise to this group of +50 analogs.

Most recently, Boutin et al. reported LC-MS/MS analysis of GluCer and its isobaric GalCer isoforms in positive ionization mode (Boutin et al., 2016). Both molecules (GluCer and GalCer) are comprised of a sugar molecule conjugated with a sphingosine molecule and a fatty acid chain. According to different lengths of fatty acid chains, it would correspond to different GluCer/GalCer couples (Figure 24.4d). MRM transitions were monitored for seven molecules: GalCer(C15:0) (molecule ion *m/z* 686.55), IS GluCer(C16:0)-d3 (molecule ion *m/z* 703.59), and five GluCer/GalCer couples, namely, GluCer/GalCer(C18:0) (molecule ion *m/z* 728.60), GluCer/GalCer(C20:0) (molecule ion *m/z* 756.64), GluCer/GalCer(C22:0) (*m/z* 784.67), GluCer/GalCer(C24:1) (molecule ion *m/z* 810.68), and GluCer/GalCer(C24:0) (molecule ion *m/z* 812.70). The same fragment ion (*m/z* 264.27), corresponding to the didehydrated sphingosine moiety, was monitored for all the molecules analyzed.

ESI negative ionization mode was also used for analyzing carbohydrate biomarkers. An LC-MS/MS method using ESI negative ionization mode coupled with graphitized carbon chromatography was reported for quantitative determination of the disaccharides derived from KS, HS, and DS in human serum and plasma (Oguma et al., 2007a). The disaccharides derived from KS, HS, and DS are highly acidic molecules and were negatively charged during ionization in MS. The mobile phase for the negative mode was a gradient elution of acetonitrile–10 mM ammonium bicarbonate buffer (pH 10) to facilitate the ionization. The mass spectrometer detected MRM transitions corresponding to fragmentation of the *m/z* 462 precursor ion to the *m/z* 97 production for Gal $\beta$ 1→4Glc-NAc(6S) and Gal(6S) $\beta$ 1→4GlcNAc(6S), *m/z* 378 to *m/z* 175 for 2-acetamido-2-deoxy-4-O-(4-deoxy-a-L-threo-hex-4-enopyranosyluronic acid)-D-glucose [ $\Delta$ DiHS-OS], *m/z* 416 to *m/z* 138 for 2-deoxy-2-sulfamino-4-O-(4-deoxy-a-L-threo-hex-4-enopyranosyluronic acid)-D-glucose [ $\Delta$ DiHS-NS], *m/z* 458 to *m/z* 97 for 2-acetamido-2-deoxy-4-O-(4-deoxy-a-L-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose [ $\Delta$ DiHS-6S], and *m/z* 354 to *m/z* 113 for chondrosine as the IS (Figure 24.2). A similar LC-MS/MS method using ESI in negative ionization mode for quantifying disaccharides derived from KS was also reported in another study by Omuga and colleagues (2007b).

A sensitive and simple method was developed by Ramsay et al. for the measurement of sulfated *N*-acetylhexosamines (HexNAcS and HexNAcS2) and sulfated *N*-acetylhexosamine/uronic acid disaccharides

(HexNAcS/uronic acid) in MPS and multiple sulfatase-deficient patients. Mass spectrometric analysis was performed in negative ionization mode using a triple-quadrupole mass spectrometer. Quantification of PMP-derivatized oligosaccharides was performed using the MRM mode. Pairs of precursor ions and product ions monitored were 630/256 (HexNAcS), 710/256 (HexNAcS2), 806/331 (HexNAcS/uronic acid), and 633/259 (Glc-NAc6S-d3 as IS). Quantification was achieved by relating the peak heights of the PMP-oligosaccharides to the peak height of the PMP-GlcNAc6S-d3 IS (Ramsay et al., 2003).

## 24.4 Conclusions

Targeted LC-MS methods play a critical role for quantitative analysis of carbohydrates from biological fluids. Analytes can be extracted from the biological matrices using multiple methods such as SPE, LLE, derivatization, and enzymatic digestion. LC separations are essential for successful determination of mono-, di- and oligosaccharides. Options include normal-phase, hydrophilic interaction, reversed-phase, reversed-phase ion-pairing, and graphitized carbon chromatographic modes. For quantitative MS analysis of carbohydrates, both MRM and TOF-MS have been employed, and both positive and negative ionization modes have been reported. TOF-MS has the advantage of performing nontargeted full mass spectrum acquisition and would potentially be utilized more frequently, especially in quantitative metabolomic biomarker studies.

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## 25

### Nucleoside/Nucleotide Biomarkers

Guodong Zhang

*Bioanalytical and Biomarker Development, Shire, Lexington, MA, USA*

#### 25.1 Introduction

Nucleosides and nucleotides are the building blocks of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Upon catabolism of DNA and RNA, the unmodified nucleosides, namely deoxyadenosine (dA), deoxyguanosine (dG), thymidine (dT), deoxycytidine (dC) from DNA, and adenosine (A), guanosine (G), uridine (U) and cytidine (C) from RNA are recycled to synthesize new DNA or RNA (Li et al., 2015). Nucleosides can also be modified by reactive oxygen species, and the modified nucleosides cannot be catalyzed by the phosphorylases to form nucleotides and are excreted from the cell into the urine. The levels of urinary modified nucleosides are fairly constant in normal healthy individuals (Gehrke et al., 1979) and not affected by age and diet. Nucleosides and nucleotides are not only involved in cellular metabolism but also act extracellular via purinergic receptors P1 and P2, to elicit a wide variety of physiological and pathophysiological responses through paracrine and autocrine signaling pathways (Contreras-Sanz et al., 2012). During pathological processes like inflammations, homeopathies and malignant diseases, the RNA turnover is faster than in normal body conditions, which results in higher nucleoside level in the blood and urine. In pathophysiological states (or sometimes in altered physiological ones such as pregnancy) the alternatively modified nucleosides (e.g., methylated) are formed and they do not undergo the recycling processes as the unmodified nucleosides as in the normal conditions. Instead, modified nucleosides are excreted intact into urine. Their abnormal levels in urine are related to increased RNA turnover and higher methyltransferase activity, both of which are caused by pathophysiological status, such as cancer (Struck-Lewicka et al., 2014). Elevated levels of nucleosides and modified nucleosides were observed in patients with physical illness, such as urogenital cancer (Nielsen et al., 2011;

Waszczuk-Jankowska et al., 2012), hepatocellular carcinoma, breast cancer, acquired immunodeficiency diseases (AIDS) and severe combined immunodeficiency diseases (SCID) (Bilbille et al., 2009; Bullinger et al., 2008a, 2008b; Chantin et al., 1996; Jeng et al., 2009). Alteration of modified nucleosides in cancer is clinically important to reveal oxidative DNA damage and RNA turnover (Nakano et al., 1993; Poulsen et al., 1998; Seidel et al., 2006). Hence, the levels of urinary nucleosides and modified nucleotides can be considered as a signal of disease status, especially as potential biomarkers for cancer diagnosis.

Nucleotides, which are composed of nucleosides and at least one additional phosphate groups, are monomer building units of polymeric nucleic acids such as DNA and RNA. Nucleotides not only are the precursors of DNA and RNA but also fulfill central roles in metabolism, such as storage and transport of metabolic energy and cellular signaling, and are incorporated into cofactors of enzymatic reactions (Seifar et al., 2009). For example, ribonucleotide triphosphates, including uridine-5'-triphosphate (UTP), cytidine-5'-triphosphate (CTP), guanosine-5'-triphosphate (GTP), and adenosine-5'-triphosphate (ATP), are the driving forces for cell growth and energy metabolism, and are involved in numerous cellular processes and show wide regulatory potential (Kochanowski et al., 2006). The tight regulation of intracellular concentrations of nucleotides, which involves a large number of enzymes, is important to maintain cellular homeostasis. Indeed, modifications in these pools have been shown to be responsible for genetic instability and cell transformation (Bester et al., 2011; Machon et al., 2014). Measurement of plasma concentrations of AMP, ADP, and ATP has provided information on their relative physiological importance in regulatory mechanisms (Borst and Schrader, 1991; Harkness et al., 1984; Mills et al., 1968). It has been found that a metabolic change in nucleotide concentrations occurs during

carcinogenesis and alterations in ATP concentration can indicate the energy status of the tumor. Adenosine-5'-monophosphate (AMP), adenosine-5'-diphosphate (ADP) and ATP are also signaling molecules related to the modulation of immune responses in cancers (Scherer et al., 2012; Schetinger et al., 2007). Plasma ATP may be associated with chronic manifestations in cystic fibrosis patients (Bergfeld and Forrester, 1992; Dietrich et al., 2000; Lader et al., 2000) and a useful biomarker for bladder dysfunction (Cheng et al., 2010; Kumar et al., 2010).

Eukaryotes, like humans, are organism with cells that contain complex structures within membranes. Signal transduction is consequently very important to moderate information through the receptors on the cell membrane. They amplify the signal coming from hormones or neurotransmitters outside the cell and cause a change in the activity of the cell. Cyclic nucleotide adenosine-3',5'-monophosphate (cAMP) and cyclic guanosine-3',5'-monophosphate (cGMP) serve as second messengers for many G-protein coupled receptors in a lot of cell types by rapidly propagating a signal from outside the cell to the appropriate molecules within the cell. These small cyclic nucleotides as second messengers can bind to cyclic nucleotide-gated ion channels and to target proteins like protein kinases, for example, protein kinase A and G (Kaupp and Seifert, 2002; Lucas et al., 2000). Modulation of intracellular cAMP/cGMP concentrations occurs by activation or inhibition of adenylyl/guanylyl cyclases, the cAMP and cGMP synthesizing enzymes. To inhibit signaling, both second messengers are degraded by different phosphodiesterases (PDEs) with more or less specificity for either cAMP or cGMP (Beavo and Brunton, 2002; Conti and Beavo, 2007; Oeckl and Ferger, 2012). Owing to the wide distribution of this second messenger system, an imbalance in its homeostatic regulation leads to a variety of pathological states and the system is targeted for treatment for several diseases such as cancer and cardiovascular, neurodegenerative, and psychiatric disorders (Reffelmann and Kloner, 2009; Reneerkens et al., 2009; Savai et al., 2010). Hence, cAMP and cGMP measurement could serve as valuable biomarkers to indicate normal biological and pathogenic processes as well as pharmacological responses to a therapeutic intervention.

## 25.2 Chromatographic Platforms for Nucleosides/Nucleotides

### 25.2.1 Challenges for Nucleosides and Nucleotides Measurement

The significant correlation between nucleosides/nucleotides and cancers or other diseases has generated a key urgent need of robust bioanalytical assays that can

reliably measure nucleosides/nucleotides in biological samples. The determination of urinary nucleosides is a challenge due to their low abundance, high polarity, and serious matrix interferences (Li et al., 2015). The main limits for nucleotide bioanalysis are poor retention and peak tailing on reversed-phase HPLC columns with conventional mobile phases due to their high polarity, low molecular weight, and strong acidic propriety. Peak tailing could result from the adsorption of the phosphate group of nucleotides to the residual hydroxyl group on the surface of reversed-phase stationary phases (Jansen et al., 2011; Zhang et al., 2014). In addition, strong anion exchange (SAX) columns that can retain and separate nucleotides are incompatible with mass spectrometry due to high concentrations of nonvolatile salt in the mobile phase (Zhang et al., 2011).

### 25.2.2 Conventional Immunoassays, CE, GC and HPLC

Over the last several decades different techniques have been reported for the measurement of nucleosides and nucleotides. The radioimmunoassay (RIA) method can be used for measurement of nucleotides. However, the nucleotides that can be quantified are limited by the lack of specific antibodies directly against nucleotides, in particular, antibodies for most of the monophosphate and diphosphate nucleotides (Piall et al., 1986). Presently the enzyme-linked immunosorbent assay (ELISA) is the most commonly used quantitative method for cAMP and cGMP. However, only one of the cyclic nucleotides can be measured in a given sample (di Villa Bianca et al., 2011; Tsugawa et al., 1991). Because of overestimation due to cross-reaction and low specificity for urinary nucleosides, immunoassays are in general not sufficient in quantitative analysis (Cooke et al., 2008). Capillary electrophoresis (CE) and capillary electrochromatography (CEC) have become attractive alternatives to measure nucleosides and nucleotides (Jiang and Ma, 2009). However, they are rarely applied for their measurement in biological samples, possibly due to the lower sensitivity and the lesser precision of the analyte migration time. Although mixtures of a variety of nucleosides can be analyzed by gas chromatography–mass spectrometry (GC–MS), derivatization to form more volatile derivatives is a prerequisite. However, the GC–MS analysis of more polar nucleotides failed (McCloskey, 1990). In addition, ATP concentration in biological samples was mainly determined by the luciferase system in clinical labs (Gorman et al., 2003, 2007). Traditional reversed-phase liquid chromatography (RP-LC) with UV or fluorescence detection has been frequently applied for quantitation of nucleosides (Davis et al., 1977), nucleotides (Caruso et al., 2004; Maessen et al., 1988), and cyclic nucleotides (Diaz Enrich et al., 2000;

Goossens et al., 1994) in biological samples. However, sample cleanup procedures in these methods are usually tedious and sensitivity is inadequate.

### 25.2.3 LC–MS/MS

Nowadays, separation techniques combined with mass spectrometry are the standard approaches for achieving assay specificity, sensitivity, and accuracy. The choice of a mass spectrometry technique mainly depends on the purpose of analysis. In untargeted analysis, all analytes that exist in a sample are determined. The sample preparation is then limited to protein precipitation (PPT), filtration, and centrifugation. In result, the sample itself is treated as a so-called fingerprint. This approach is very useful, for instance, in searching for new metabolites that can distinguish the two studied populations (e.g., healthy vs. patients) and play a potential role in a clinical diagnosis. In case of untargeted approach which is in fact a qualitative study, high-resolution mass spectrometry analyzer is needed in order to have the required specificity for the analytes (Orbitrap or time of flight (TOF)). Contrary to untargeted approach, targeted analysis (such as nucleosides or nucleotides) is focused on quantitative analysis and the employed mass spectrometry technique should be sensitive and specific enough to allow measurement of the changes between healthy and patients (Cohen et al., 2010; Kamčeva et al., 2015; Struck et al., 2011, 2013; Struck-Lewicka et al., 2014). The most often used platforms are triple quadrupole mass spectrometry. For targeted analysis, the assay should be evaluated from various analytical perspectives including recovery, precision, accuracy, sensitivity, limit of quantification, matrix effects, and matrix stability of analytes. So far, LC–MS is widely utilized to measure nucleosides and nucleotides in biological samples (Table 25.1). In this paper, an overview of the most commonly used LC–MS/MS assays for nucleosides and nucleotides measurement will be described with focus on techniques for the sample preparation, chromatography conditions, and evaluation of nucleosides and nucleotides as cancer biomarkers.

## 25.3 Bioanalytical Methodologies

### 25.3.1 Sample Preparation Strategies

Sample preparation procedure is often the most important step for bioanalytical assays. To obtain well-purified and/or concentrated samples, the physicochemical properties and structures of the analytes need to be taken into account (Struck et al., 2011; Tuytten et al., 2007). Solid-phase extraction (SPE) is widely used for the extraction and pre-concentration of nucleosides in urine. The most often used sample preparation technique is phenylboronic acid (PBA) gel as a sorbent bed (Davis et al., 1977; Kammerer et al.,

2005; Struck et al., 2013; Zhao et al., 2006). This type of extraction is selective to vicinal hydroxyl groups that exist in ribose moiety attached to nucleobase moiety and relies on changes of pH. Under basic conditions ( $\text{pH} > 8$ ) the *cis*-diol groups of nucleosides bind to the PBA sorbent and all interferences that occur in the extracted sample can be flushed. Afterward, by changing pH from basic to acidic ( $\text{pH} < 3$ ), the *cis*-diol groups are being released from the sorbent and immediately eluted. Recently, boronate-decorated polyethylenimine-grafted hybrid magnetic nanoparticles (Li et al., 2013) and zirconium-doped magnetic microspheres were developed to extract and enrich nucleosides (Fan et al., 2016). The main disadvantage of this type of extraction is that the phenyl-boronic acid sorbent is not selective to deoxynucleosides due to a lack of *cis*-diol group. Alternatively, cation-exchange extraction and Oasis HLB cartridge were applied to extract nucleosides (Teichert et al., 2011; Tuytten et al., 2007). HLB extraction cartridges have been employed for extraction of purine nucleosides from urine for LC–MS/MS analysis (Li et al., 2009). Oasis MCX cartridge was successfully applied to extract 2-deoxynucleosides, which cannot be purified by PBA SPE (Hsu et al., 2009, 2011). The LC–MS/MS approach with selective reaction monitoring (SRM) and MCX sample preparation allowed for the sensitive determination of urinary levels of 3 nucleosides, cytidine, 3-methylcytidine, and inosine, which were found significantly higher in breast cancer patients than in normal controls ( $p < 0.01$ ). In another study, the mean levels of five urinary nucleosides (adenosine, cytidine,  $N^2, N^2$ -dimethylguanine, 8-hydroxy-2'-deoxyguanosine and uridine) were significantly higher in the patients with colorectal cancer than in the healthy adults (Hsu et al., 2009).

Modern analytical techniques make it possible to develop online extraction of nucleosides by directly injecting urine samples to online extraction column which improves automation of the sample preparation process and minimizes sample manipulation. The proposed analytical procedure was fully automated and was based on using two coupled columns: a first column to clean and concentrate nucleotides from the biological matrix, and a second column to perform the analytical chromatographic separation (Hagemeier et al., 1983; Machon et al., 2014). Online combination of aprotic boronic acid chromatography with HILIC–MS platform was reported to measure nucleosides in urine samples (Tuytten et al., 2008). A highly polar restricted-access material (RAM) based on an *N*-vinylacetamide copolymer was developed, and its combination with ZIC-HILIC–MS/MS provides a convenient approach for online nucleosides extraction and matrix removal (Rodríguez-Gonzalo et al., 2011). In another example, online extraction by *n*-vinylacetamide copolymer was applied to efficiently remove interferences to determine urinary nucleosides (Cho et al., 2009) (Table 25.1).

**Table 25.1** Examples of quantitation of nucleosides and nucleotides in biological samples using LC-MS/MS techniques.

Analytes	Biological matrices	Sample preparation	Column	Chromatography	Detection limit	Calibration range	Biomarker application	Reference
C, m3C, m1A, A, G, dG, 8-OHdG, NNGua, I	Human urine	MCX SPE	Atlantis d C <sub>18</sub>	RP	0.1 µg/mL	0.1–20 µg/mL	Breast cancer	Hsu et al. (2011)
C, U, A, G, m1A, 8-OHdG, NNGua, 5-OHDU	Human urine	Column-switching	<i>n</i> -Vinylacetamide copolymer and C <sub>18</sub>	RP	0.2 nmol/mL	0.2–100 nmol/mL	Breast cancer	Cho et al. (2009)
C, Pseu, U, m3C, 3mU, 5mU, A, I, 6mA, X, N2mG, NNGua	Human urine	PBA SPE	C <sub>18</sub>	RP	0.96, 1.26, 2.25, 1.44, 1.29, 0.42, 0.87, 1.65, 1.14, 1.23, 1.38, 1.26 µM	1–200, 2.5–200, 2.5–400 µM	Urogenital track cancers	Struck et al. (2013)
U, 8-OHdG, I, 8-OHG, A, G, 7mGua, m1A, 7mG	Human urine	Online-RAM extraction	ZIC-HILIC	HILIC	30.0 ng/mL	30–10,000 ng/mL	Cancers	Rodríguez-Gonzalo et al. (2011)
A, C, I, m1A, m3C, U, dG	Human urine	MCX SPE	Atlantis d C <sub>18</sub>	RP	0.1, 0.1, 0.25, 0.025, 0.50, 0.25, 0.1 µg/mL	0.1–20, 0.25–20, 0.025–20, 0.05–20 µg/mL	Hepatocellular carcinoma	Jeng et al. (2009)
C, m3C, m1A, A, I	Human urine	MCS SPE	Atlantis d C <sub>18</sub>	RP	0.1, 0.05, 0.025, 0.1, 0.25 µg/mL	0.1–20, 0.05–20, 0.025–20, 0.1–20, 0.25–20 µg/mL	Gastric cancer	Lo et al. (2014)
A, G, U, C	Human urine	Acetone derivatization	BEH phenyl	RP	1.00 ng/mL	1–1000 ng/mL	NA	Li et al. (2015)
AMP, ADP, ATP	Human plasma	PPT	Amino column	DEA-HFIP IP-HILIC	10, 10, 2.0 ng/mL	10–1000, 10–1000, 2–200 ng/mL	NA	Zhang et al. (2014)
AMP, ADP, ATP	Cultured cells	Perchloric acid PPT	C <sub>8</sub>	DMHA IP-RP	0.1, 2.0, 2.5 µg/mL	0.1–20, 2.0–20, 2.5–20 µg/mL	NA	Qian et al. (2004)
AMP, ADP, ATP	HepG-2 cell	Perchloric acid PPT	PGC	RP	0.22, 0.59, 0.49 µg/mL	0.22–57.8, 0.59–117.37, 0.49–98.81 µg/mL	NA	Wang et al. (2009)
ATP, CTP, GTP, UTP, dATP, dCTP, dGTP, dTTP	Human PBMC	SPE	PGC	IP-RP	5 pmol for nucleotides, 0.125 pmol for deoxynucleotides	0.25–312.5 pmol, 0.125–20.8 pmol	NA	Kamčeva et al. (2015)
UMP, GMP, AMP, dCTP, ATP	Serum, urine, tissue	PPT	HSST3	IP-RP	NA	NA	Pancreatic cancer or arthritic	Michopoulos et al. (2014)
AMP, ADP, ATP	Rat liver	PPT	PGC	RP	1.6 ng/mL	10–5000 ng/mL	NA	Jiang et al. (2012)
AMP, ADP, ATP, CDP, CTP, GDP, GTP, UDP, UTP	Rat kidney	PPT	C <sub>18</sub>	DBAF IP-RP	2, 2, 1, 1, 2, 2, 2, 1, 2 µM	2–500, 2–500, 1–250, 1–250, 2–250, 2–250, 2–250, 1–250, 2–250 µM	NA	Klawitter et al. (2007)

ATP, CTP, GTP, UTP, dATP, dCTP, dTTP	Cells	PPT	Altantis TS	TEA-HFIP IP-RP	0.5, 1, 5 nM	0.5–500, 1–500, 5–500 nM	NA	Wu et al. (2015)
ATP, ADP, AMP, GTP, GDP, GMP, CTP, CDP, CMP, UTP, UDP, UMP	Human cancer cells	TCA PPT	C <sub>18</sub>	DEA-HA IP-RP	77, 6.5, 7.5, 25, 1.8, 1.2, 6.4, 1.75, 2.5, 7, 5, 1.4 pmol	77–9240, 6.5–650, 7.5–750, 2.5–1000, 1.8–142, 1.2–96, 6.4–640, 1.75–140, 2.5–200, 7–350, 5–200, 1.4–54 pmol	Human cancer cells	Zhang et al. (2011)
cAMP, AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP, UTP	Cells	Boiling aqueous ethanol	XTerra C <sub>18</sub>	DBAA IP-RP	0.04, 0.03, 0.12, 0.12, 0.31, 0.06, 0.06, 0.62, 0.03, 0.12, 0.03, 0.03, 0.06, 0.31, 0.12, 0.12 pmol	0.25–100 μM	NA	Seifar et al. (2009)
dGTP, dATP, dTTP, dCTP	PMBCs	PPT	Supelcolgel ODP-50	DMH IP-RP	0.3 pmol for dTTP and dCTP, 0.4 pmol for dATP and dGTP	0.3–20 pmol	HIV-infected patients	Henneré et al. (2003)
cGMP, cAMP	Mice plasma, CSF, brain tissue	Perchloric acid PPT	MetaSil AQ C <sub>18</sub>	RP	50 pM	0.5–500 nM	PDE4 inhibitor in mice	Oeckl et and Ferger (2012)
cGMP, cAMP	Human plasma	HILIC SPE	Zorbax SB-C18	RP	0.15 ng/mL	0.15–20 ng/mL	NA	Van Damme et al. (2012)
cGMP	Human plasma	PPT	MetaSil AQ C <sub>18</sub>	RP	0.5 ng/mL	0.5–20 ng/mL	NA	Zhang et al. (2009)

3mU, 3-methyluridine; 5mU, 5-methyluridine; 5-OHdU, 5-hydroxymethyl-2'-deoxyuridine; 6mA, 6-methyladenosine; 7mG, 7-methyl-guanine; 7mGua, 7-methyl-guanine; 8OHdG, 8-hydroxy-2-deoxyguanosine; 8-OHG, 8-hydroxy-guanosine; A, adenosine; ADP, adenosine-5'-diphosphate; AMP, adenosine-5'-monophosphate; ATP, adenosine-5'-triphosphate; C, cytidine; cAMP, adenosine cyclic-3',5'-monophosphate; CDP, cytidine-5'-diphosphate; cGMP, 3'5'-cyclic guanosine monophosphate; CMP, cytidine-5'-monophosphate; CTP, cytidine-5'-triphosphate; DBAA, dibutylammonium acetate; DBAF, dibutylammonium formate; DEA-HFIP, diethylamine hexafluoroisopropanol; dG, 2-deoxyguanosine; DMH, dimethylhexamine; DMHA, dimethylhexylamine; G, guanosine; GDP, guanosine-5'-diphosphate; GMP, guanosine-5'-monophosphate; GTP, guanosine-5'-triphosphate; HILIC, hydrophilic interaction liquid chromatography; I, inosine; IDP, inosine-5'-diphosphate; IMP, inosine-5'-monophosphate; IP-HILIC, ion-pair hydrophilic interaction liquid chromatography; IP-RP, ion-pair reversed-phase; ITP, inosine-5'-triphosphate; m1A, 1-methyladenosine; m3C, 3-methylcytidine; N2mG, N-2-methylguanosine; NA, not available; NNGua, N<sup>2</sup>,N<sup>2</sup>-dimethylguanine; PBA, phenylboronic acid; PGC, porous graphitic column; PPT, protein precipitation; Pseu, pseudouridine; RP, reversed phase; SPE, solid-phase extraction; TCA, trichloroacetic acid; TEA-HFIP, triethylamine hexafluoroisopropanol; UDP, uridine-5'-diphosphate; UMP, uridine-5'-monophosphate; UTP, uridine-5'-triphosphate; X, xanthosine.

The analysis of nucleotides has been performed in various matrices such as whole blood, plasma, erythrocytes, peripheral blood mononuclear cells (PBMC), cultured cells, cerebrospinal fluid (CSF), or tissue (Kamčeva et al., 2015; Seifar et al., 2009; Uehara et al., 2009; Zhang et al., 2014). These matrices contain enzymes involved in the purines and pyrimidines metabolism. The first step in sample preparation is to lyse the cells or organelles and block the metabolism of nucleotides immediately by inactivation of the enzymes. To do this, the extraction was generally performed at low temperature. High concentration of perchloric acid (PCA) or trichloroacetic acid (TCA) solution was commonly applied to extract nucleotides from biological samples (Zhang et al., 2011; zur Nedden et al., 2009). Unfortunately, the injection solution with high concentration of PCA or TCA exhibited significant ion suppression for nucleotides in the LC-MS/MS approach (Zhang et al., 2014). Alternatively, direct protein precipitation (PPT) using acetonitrile can be applied, but it only exhibited less than 40% of extraction efficiency for ATP from plasma. Instead, the extraction efficiency was significantly enhanced to above 67.0% in plasma and above 92.9% in 200-fold diluted blood using methanol PPT procedure (plasma/methanol at 1:2 volume ratio). It is very interesting that methanol generates much higher extraction efficiency for nucleotides in plasma than acetonitrile. It could be hypothesized that acid compounds like nucleotides have high protein binding propensity and methanol could release nucleotides from protein binding sites more efficiency than acetonitrile (Zhang et al., 2014). Taken altogether, the best extraction procedure proposed was the addition of methanol cooled at -20°C followed by sonication and hexane extraction for lipid removal (Cordell et al., 2008). Oasis WAX SPE was also applied to extract nucleotides from biological samples (Cohen et al., 2009; Kamčeva et al., 2015). However, SPE is time consuming and may cause less accuracy compared to direct PPT procedure (Cohen et al., 2009). For cAMP and cGMP, PPT and HILIC SPE were in general deployed for extraction (Oeckl and Ferger, 2012; Van Damme et al., 2012; Zhang et al., 2009) (Table 25.1).

### 25.3.2 Chromatographic Strategies for LC-MS/MS Assays

Nowadays, chromatographic techniques are well established and commonly used in nucleoside bioanalysis. Owing to their physiochemical properties, nucleosides are mainly analyzed by reversed-phase HPLC (RP-HPLC) with a C<sub>18</sub> column (Cho et al., 2009; Hsu et al., 2009, 2011; Struck et al., 2013). Hydrophilic interaction chromatography (HILIC) is also worth considering. Its characteristic feature is the use of a polar stationary phase (e.g., silica, cyano, amino, or amide) and a highly organic

mobile phase with a smaller amount of aqueous solvent. In contrast to the composition of the mobile phase widely used in RP-HPLC, the aqueous solvent in HILIC is classified as a stronger eluent than the organic modifier. Hence, the method can retain highly polar analytes that would not be retained by RP-HPLC. It makes it possible to omit the evaporation step following the usually performed extraction because the organic extract (e.g., PPT) may be directly injected to HILIC-MS/MS system (Jian et al., 2010). A new approach was developed for nucleoside measurement with an aprotic boronic acid precolumn by online extraction, which was switched to the HILIC column with stationary phase of polyhydroxyethyl aspartamide (Tuytten et al., 2008). The mobile phase for the precolumn was 100% acetonitrile. For the HILIC column, mobile phase A was 10 mM ammonium formate in 95:4.8:0.2 acetonitrile/water/formic acid, and mobile phase B was 10 mM ammonium formate in 50:49.8:0.2 acetonitrile/water/formic acid. In another example, ZIC-HILIC combined with online restricted-access material (RAM) extraction was reported to determine nucleosides in human urine with limits of detection from 0.1 to 1.3 ng/mL (Rodríguez-Gonzalo et al., 2011).

Nucleotides are chemical compounds with two different regions: a negatively charged phosphate group(s) ( $pK_a$  around 1), and the ribose basic group, a less polar one. Their polarity increases with the number of phosphate group. Unlike nucleosides, it is extremely challenging to retain nucleotides, especially triphosphates nucleotides, on traditional RP-HPLC conditions due to their high polarity. In most methods for the simultaneous determination of nucleoside mono-, di- and triphosphates, RP-HPLC was rarely used and shown to be inadequate.

Ion-pair (IP) chromatography is in principle the most suitable method for the determination of nucleotides. An IP chromatography and an anion exchange (AE) chromatography were employed for the determination of nucleotides and sugar nucleotides. The study showed that the separation for the nucleotides was satisfactory with both methods and the analysis times were similar (Tomiya et al., 2001). However AE chromatography suffered from a poor compatibility with mass spectrometry (Seifar et al., 2009). In contrast, IP chromatography is considered as the most appropriate methods in terms of compatibility with the MS detection for analysis of charged compounds. The separation is based on the formation of ion pairs between the negatively charged nucleotide and the positively charged ion-pairing reagents (alkylamines) in the mobile phase. The tetrabutylammonium salts was successfully used for HPLC analysis of mono-, di- and triphosphates nucleoside coupled with UV detection mode (Yeung et al., 2008). However, tetrabutylammonium salts were inherently nonvolatile and therefore incompatible with electrospray mass spectrometer (Luo et al., 2007). One solution was to use more volatile amines

such as triethylamine (TEA) (Cichna et al., 2003), diethylamine, tributylamine (Luo et al., 2007), dibutylammonium salt (Klawitter et al., 2007; Seifar et al., 2009), dimethylhexylamine (DMHA), or hexylamine (HA) (Cordell et al., 2008; Coulier et al., 2006; Tuytten et al., 2002; Zhang et al., 2011). The concentration of ion-pairing agent in the mobile phase and the pH were critical and had to be optimized to obtain good retention and peak shapes, ion suppression and minimized contamination when spectrometric mass detection was used. The concentration of trialkylamine used as ion-pairing agent could be reduced by increasing the length of the alkyl chain and thus promoting interactions between the hydrophobic stationary phase and the ion-pairing agent. One solution for reducing contamination of the electrospray ionization (ESI) source and mass spectrometer caused by sample matrix and ion-pairing reagent present in the mobile phase was the decrease in flow of the mobile phase by using a splitter before the entrance of the mass spectrometer (Coulier et al., 2006; Seifar et al., 2009). Another way was to use column miniaturization (Cichna et al., 2003). A nano-LC-ESI-MS/MS method was described for quantification of phosphorus metabolites in cells (Uehara et al., 2009). Good separation of compounds was achieved with 40- to 100-fold increase of sensitivity compared with a semi-micro-LC-MS/MS.

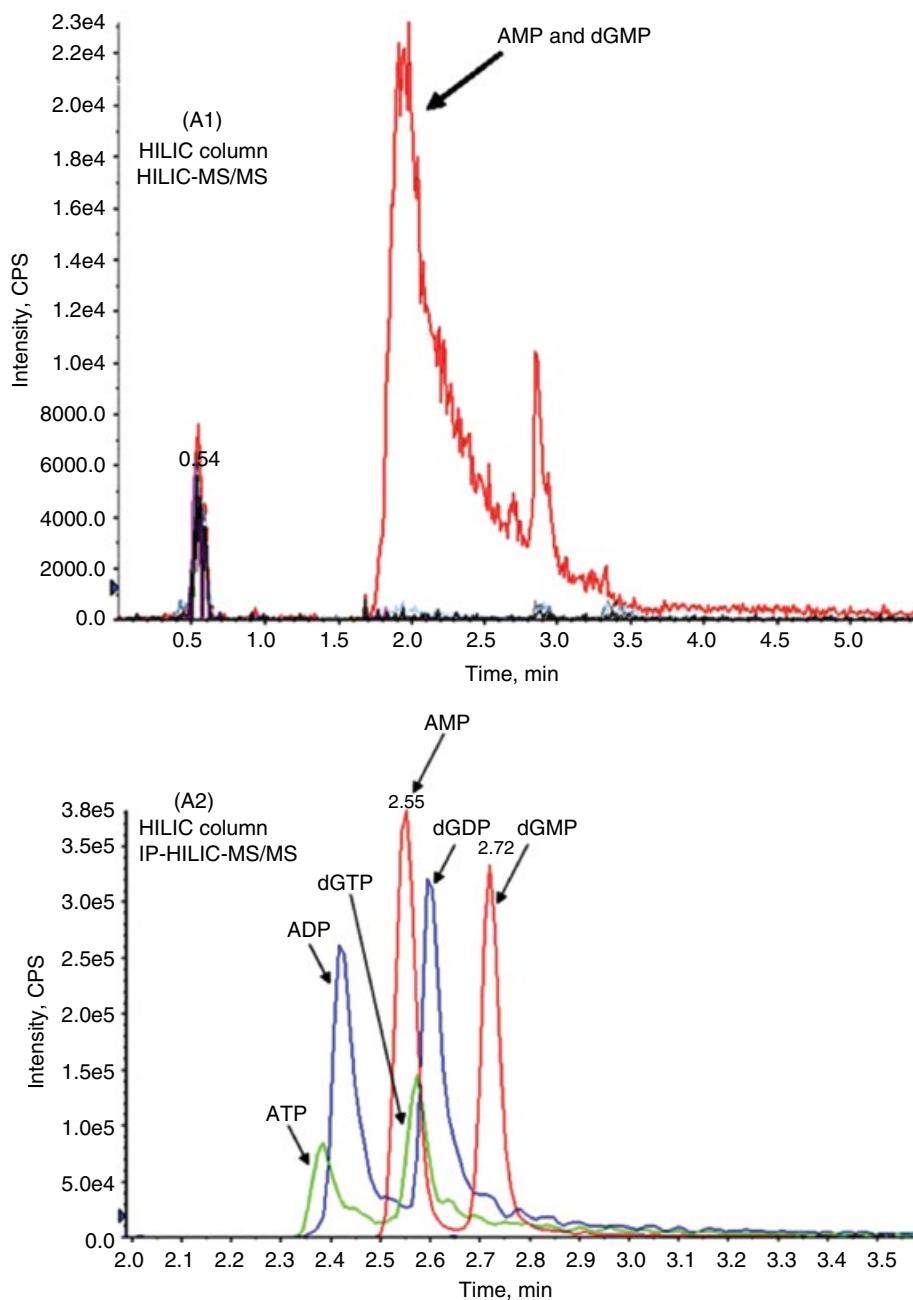
Another way to achieve the separation of nucleotides was the use of carbon column (Wang et al., 2009; Xing et al., 2004). The physical and chemical stability of graphitic carbon (PGC) columns allows its applications for polar compounds that are difficult to retain on silica-based packing materials. The retention mechanisms on PGC are complex, involving hydrophobic interactions and electrostatic interactions. The PGC is involved in  $\pi-\pi$  interactions and dispersive interactions with aromatic solutes. Retention increases as the hydrophobicity of the molecule increases. Furthermore, the graphitic carbon surface acts as a Lewis base toward polar solutes. When an electrostatic charged polar analyte approaches the graphite surface, charge-induced dipole at the graphite surface is generated, increasing the attraction between the analyte and support. Furthermore, PGC has the advantage to tolerate a broad range of pH (0–14) (Hanai, 2003). A general LC-MS method utilizing PGC column was developed for the analysis of nucleosides and their mono-, di- and triphosphates. It was showed that the concentration of ammonium acetate was critical for elution of nucleotides and for maximizing MS detector sensitivity. The presence of diethylamine (DEA) reduced retention of monophosphates and enhanced their MS signal intensity (Xing et al., 2004). One disadvantage of PGC column was difficult desorption of non-polar compounds adsorbed on the surface, which may contribute to variation in retention times between analytical runs (Kamčeva et al., 2015).

Recently, HILIC technique was applied widely for the separation of highly polar compounds, including nucleotides (Buszewski and Noga, 2012; Marrubini et al., 2010). Several HILIC-MS/MS assays were reported to determine nucleotides (Bajad et al., 2006; Johnsen et al., 2011). Nevertheless, most of the above HILIC-MS/MS assays only provided limited separation capacity and sensitivity with relative long run time (above 30 min). Traditional HILIC mode using Atlantis bare silica HILIC column or NH<sub>2</sub> column and 50 mM ammonium formate containing 0.1% formic acid (v/v) in mobile phases (pH 4.0) were assessed, but only broad peaks with tailing for AMP and dGMP were obtained with the bare silica HILIC column, and ADP, dGDP, ATP, and dGTP were not detected. Also, no peaks were observed when utilizing the NH<sub>2</sub> column. It could be explained, to some extent, by the hypothesis that the polar surface of bare silica stationary phase or NH<sub>2</sub> column has strong hydrophilic interaction with extremely polar acidic nucleotides, which contributed to tailing, broad peak, or no peaks, even though a high percentage of aqueous phase (70%; v/v) was utilized to elute analytes. In addition to hydrophilic interaction, the hybrid anion exchange on NH<sub>2</sub> column is also too strong for elution of nucleotides by using 50 mM ammonium formate containing 0.1% formic acid (v/v) at pH 4.0 without high salt concentrations that would be incompatible with LC-MS. Recently, another HILIC-MS/MS assay was described to determine nucleotides utilizing NH<sub>2</sub> column with high pH mobile phase (pH 9.45) (Bajad et al., 2006). However, the retention time was fairly long for ATP at around 30 min and the sensitivity (LOD) was 500 ng/mL for ATP and 100 ng/mL for ADP, respectively, which was not low enough to detect nucleotides in plasma samples.

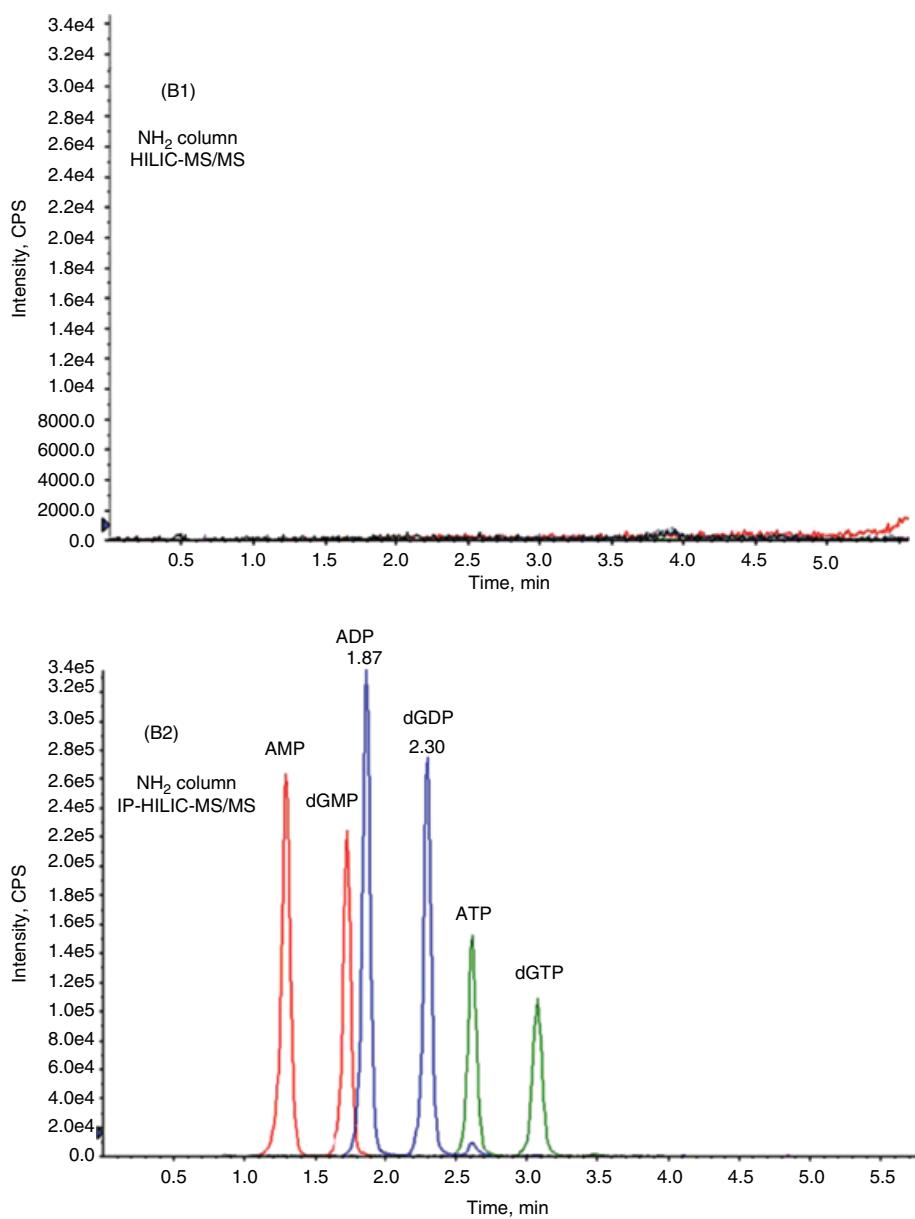
Taking the extremely polar and acidic nature of nucleotides into account, a novel chromatography mechanism, IP-HILIC has emerged as a promising alternative to IP-RP-LC-MS/MS with shorter run time, higher sensitivity, and improved chromatographic separation capacity for nucleotides. A novel IP-HILIC-MS/MS by utilizing DEA as ion-pairing reagent was developed to measure nucleotides in biological samples (Zhang et al., 2014). It was speculated that there is formation of ion pairs of nucleotides with DEA, but not to the extent to impact the hydrophilic retention because DEA has a short alkyl chain as compared to other ion paring reagents such as tributylamine or hexylamine, which form more hydrophobic complexes with nucleotides. The peak shape of nucleotides was improved significantly because DEA could neutralize the hydroxyl group on the surface of stationary phases. The separation was dramatically improved on the bare silica HILIC column and the NH<sub>2</sub> column. In addition, DEA-hexafluoroisopropanol (HFIP) buffer at pH 8.9 to some extent neutralized the surface of NH<sub>2</sub> column ( $pK_a$  9.8), reduced the anion exchange capacity, and contributed to short

retention for nucleotides as well. Another advantage of DEA is its lower boiling point, 55–56°C compared to tributylamine (216°C) and hexylamine (131–132°C). From previous publications, lower limit of quantitation (LLOQ) of ATP at 100 nM (equal to 50.7 ng/mL) was obtained using tributylamine (Luo et al., 2007) in the mobile phases and 20.0 ng/mL of LOD for ATP was reported using hexylamine (Coulier et al., 2006). For the

novel IP-HILIC-MS/MS assay utilizing DEA-HFIP buffer in the mobile phases, LLOQ at 2.00 ng/mL (3.9 nM or 19.7 fmol on column) for ATP was successfully obtained, which is at least 10-fold more sensitive than above reported LC-MS/MS assays with standard columns and instrumentation except nano-LC-MS/MS assay (Kiefer et al., 2011). It could be speculated that mobile phases with lower boiling point result in higher



**Figure 25.1** MRM chromatograms of standard solution (200 ng/mL for each) of AMP, ADP, ATP, dGMP, dGDP and dGTP on bare silica HILIC column and NH<sub>2</sub> column using HILIC-MS/MS or IP-HILIC-MS/MS platform. (A1) HILIC-MS/MS on bare silica column, (A2) IP-HILIC-MS/MS on bare silica column, (B1) HILIC-MS/MS on NH<sub>2</sub> column and (B2) IP-HILIC-MS/MS on NH<sub>2</sub> column. Source: Zhang et al. (2014). Reproduced with permission of Elsevier.



**Figure 25.1** (Continued)

ionization efficiency and better sensitivity for analytes in ESI. In addition, DEA is much easier to remove from the instrument compared to tributylamine and hexylamine, which require extensive cleaning of the instrument to allow use of positive ion mode ESI-MS detection.

For isobaric isomers such as AMP and dGMP, ADP and dGDP, ATP and dGTP with identical MRM transitions, a baseline chromatographic separation is necessary to reduce the possibility of interference between them. However, chromatographic separation of these isobaric isomers has been particularly challenging (Cohen et al., 2009; Coulier et al., 2006). Recently, an

ion-pair reversed-phase LC-MS/MS was described to separate these isobaric isomers with a run time of around 40 min (Zhang et al., 2011). In contrast, IP-HILIC-MS/MS platform successfully separated them with high sensitivity using bare silica HILIC column or NH<sub>2</sub> column within 7.5 min, providing a rapid and sensitive approach to simultaneously determine these isobaric isomers. Under the IP-HILIC condition, it was observed that ATP and dGTP exhibited tailing peaks on bare silica HILIC column while showing good peak shape on NH<sub>2</sub> column (Figure 25.1). This could be due to the additional hybrid anion exchange mechanism on the NH<sub>2</sub> column which

contributes to improved chromatographic retention and separation compared to bare silica column. The IP-HILIC separation on the NH<sub>2</sub> column is a mixed mode mechanism combining hydrophilic interaction with anion exchange. HFIP (100 mM) in mobile phase in addition to DEA played another key factor in improving the peak shape and sensitivity. HFIP is a weak acid with a low boiling point, and it is widely used for oligonucleotide bioanalysis to enhance sensitivity for negative mode (Apffel et al., 1997; Zhang et al., 2007). DEA-HFIP buffer as ion-pair HILIC mobile phase was applied to determine nucleotides in biological samples with high separation capacity, sensitivity, and short run time. It should be noted that the sensitivity of nucleotides was decreased dramatically if formic acid or acetic acid was added to mobile phase. In contrast, 100 mM HFIP and 0.5% DEA (v/v) in mobile phase at pH 8.9 were optimal to provide the highest sensitivity and chromatographic separation capacity for nucleotides. Recently, IP-HILIC XBridge-Amide was found to be a suitable column for the separation of nucleotides utilizing TEA-HFIP mobile phase (Mateos-Vivas et al., 2015) (Table 25.1).

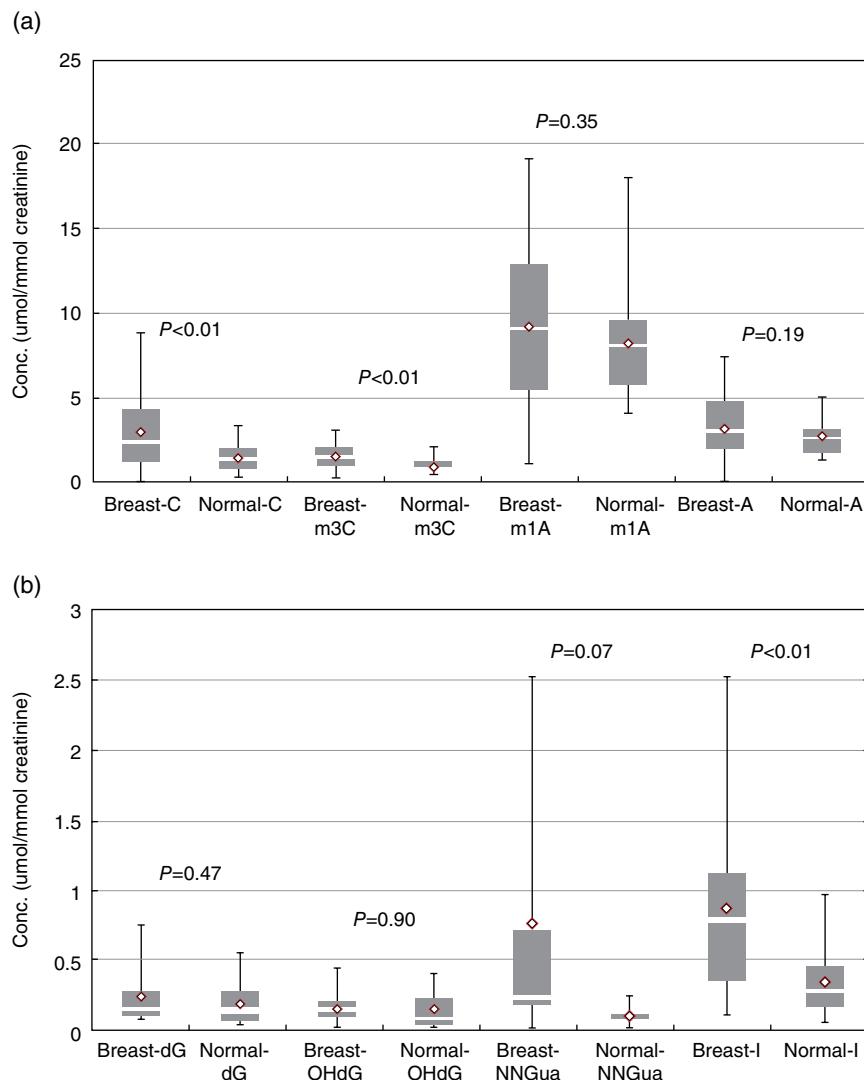
## 25.4 Nucleoside/Nucleotide Biomarkers and Case Studies

Breast cancer is the most common female cancer. In one study, urinary nucleoside levels in female breast cancer patients ( $n=36$ ) were measured by LC-MS/MS to evaluate the diagnostic value of nucleosides as potential tumor markers (Hsu et al., 2011). Sample preparation was performed using a 96-well cation-exchange SPE. Cation-exchange allows for the comprehensive purification of modified nucleosides, such as 2-deoxynucleosides, that are not purifiable by PBA-based SPE. The method was shown to be more specific and sensitive than traditional UV detection. The results demonstrated urinary levels of three nucleosides, cytidine, 3-methylcytidine, and inosine were significantly higher in breast cancer patients than in normal controls ( $p<0.01$ ). The discriminative powers of cytidine, 3-methylcytidine, and inosine were 58, 58, and 62%, respectively (Figure 25.2). The research is the first to identify 3-methylcytidine as an abundant nucleoside in breast cancer urine in addition to cytidine and inosine.

Measurement of endogenous ATP, ADP, and AMP in human plasma and blood is very challenging due to extremely high polarity, chromatography issues, and low concentration. A novel and highly sensitive IP-HILIC-MS/MS method was developed for quantitation

of highly polar acid metabolites like AMP, ADP, and ATP nucleotides in human plasma and blood (Zhang et al., 2014). A mobile phase based on DEA and HFIP and an aminopropyl (NH<sub>2</sub>) column were applied for chromatographic separation of AMP, ADP, and ATP. This novel IP-HILIC mechanism could be hypothesized by the ion-pairing reagent (DEA) in the mobile phase forming neutral and hydrophilic complexes with the analytes. The IP-HILIC-MS/MS assay for adenine nucleotides was successfully validated with satisfactory linearity, sensitivity, accuracy, reproducibility, and matrix effects. The lower limit of quantitation (LLOQ) at 2.00 ng/mL obtained for ATP showed at least 10-fold higher sensitivity than previous LC-MS/MS assays. The endogenous level ranged from 49.6 to 585 ng/mL for AMP, 54.5–795 ng/mL for ADP and 7.82–819 ng/mL for ATP in plasma. The endogenous level was in the range of 3370–18,500 ng/mL for AMP, 38,400–71,900 ng/mL for ADP, and 82,800–246,000 ng/mL for ATP in blood, respectively. Endogenous level of adenine nucleotides in human blood is above 50-fold higher than that in human plasma because the concentration in blood included both intracellular and extracellular portions which are extracted using methanol crash (Figure 25.3).

cGMP and cAMP are essential second messenger molecules. They are involved in signal transduction within cells, in physiological functions such as neurotransmission and in the modulation of cell growth and differentiation of organisms. A quantitative LC-MS/MS method-based HILIC SPE on silica was developed and applied to both plasma and tissue samples (Van Damme et al., 2012). The stable isotope labeled internal standards <sup>2</sup>D<sub>1</sub>,<sup>15</sup>N<sub>3</sub>-3',5'-cGMP and <sup>13</sup>C<sub>10</sub>,<sup>15</sup>N<sub>5</sub>-3',5'-cAMP were added prior to the sample preparation to ensure high precision and accuracy. The samples were analyzed by RP-LC. Negative electrospray (-ESI)-MS/MS was used to selectively monitor several transitions of each analyte. The method for the analysis of 3',5'-cAMP and 3',5'-cGMP in plasma was validated in the range of 0.15–20 ng/mL ( $R^2=0.9996$  and 0.9994 for 3',5'-cAMP and 3',5'-cGMP, respectively) (Figure 25.4). Basal plasma concentrations for 15 healthy human patients determined with this method varied between 4.66 and 9.20 ng/mL for 3',5'-cAMP and between 0.30 and 1.20 ng/mL for 3',5'-cGMP, with precisions better than 9.1%. 3',5'-cGMP and 3',5'-cAMP together with their 2',3'-isomers were also determined in a semi-quantitative way in animal tissues. The structures of the isomers were confirmed by analysis with LC-high resolution TOF MS and subsequently by comparison of retention times with standards.



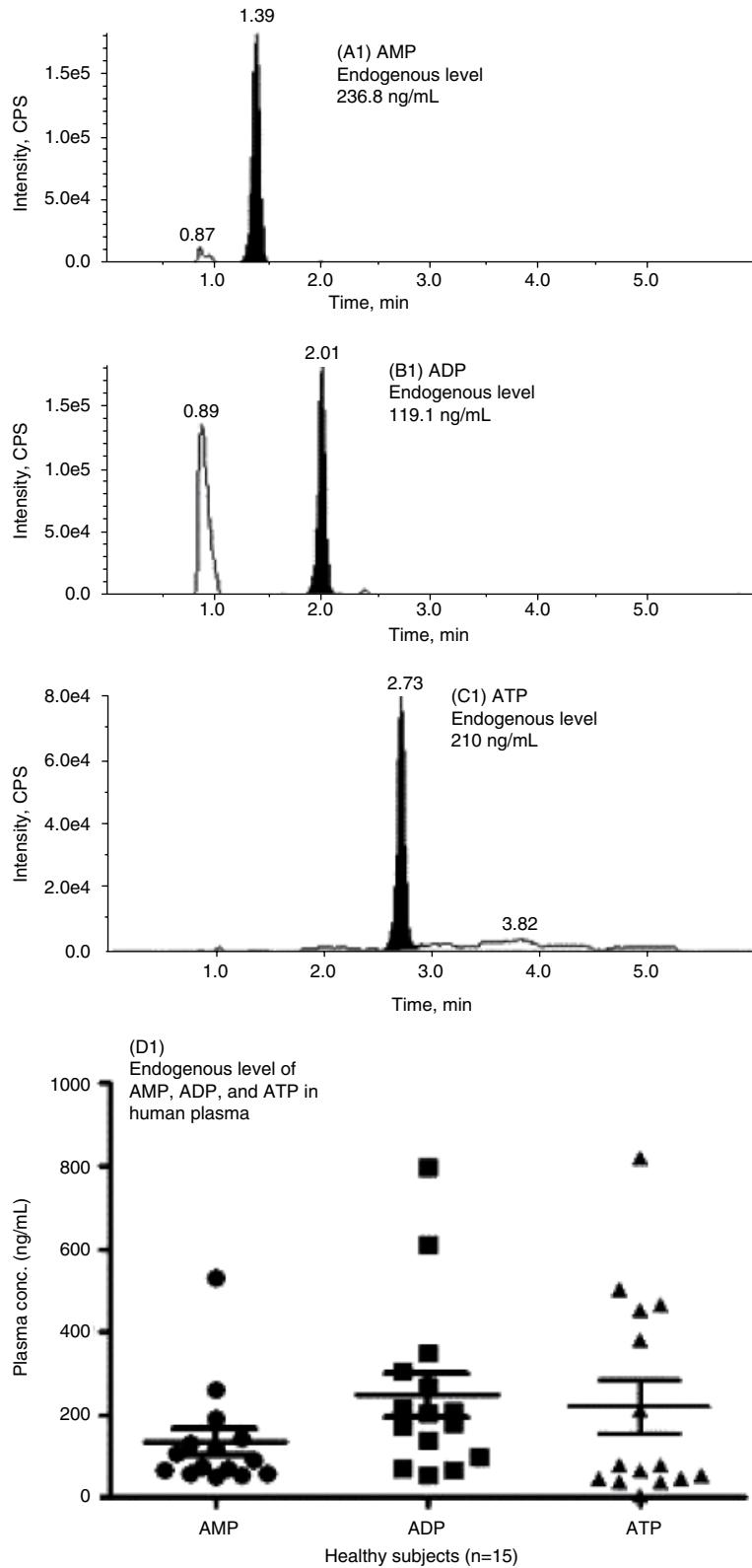
**Figure 25.2** Box plots of urinary nucleosides (A) cytidine (C), 3-methylcytidine (m3C), 1-methyladenosine (m1A), adenosine (a) and (b) 2-deoxyguanosine (dG), 8-hydroxy-2-deoxyguanosine (OHdG),  $N^2,N^2$ -dimethylguanosine (NNGuA), inosine (I). The bottom and top of the box represent the 25th and 75th percentile (the lower and upper quartiles, respectively), the band near the middle of the box is the 50th percentile, and the square circle represents the average. In addition, the ends of the whiskers represent the maximum and minimum values. Source: Hsu et al. (2011). Reproduced with permission of Elsevier.

## 25.5 Conclusion

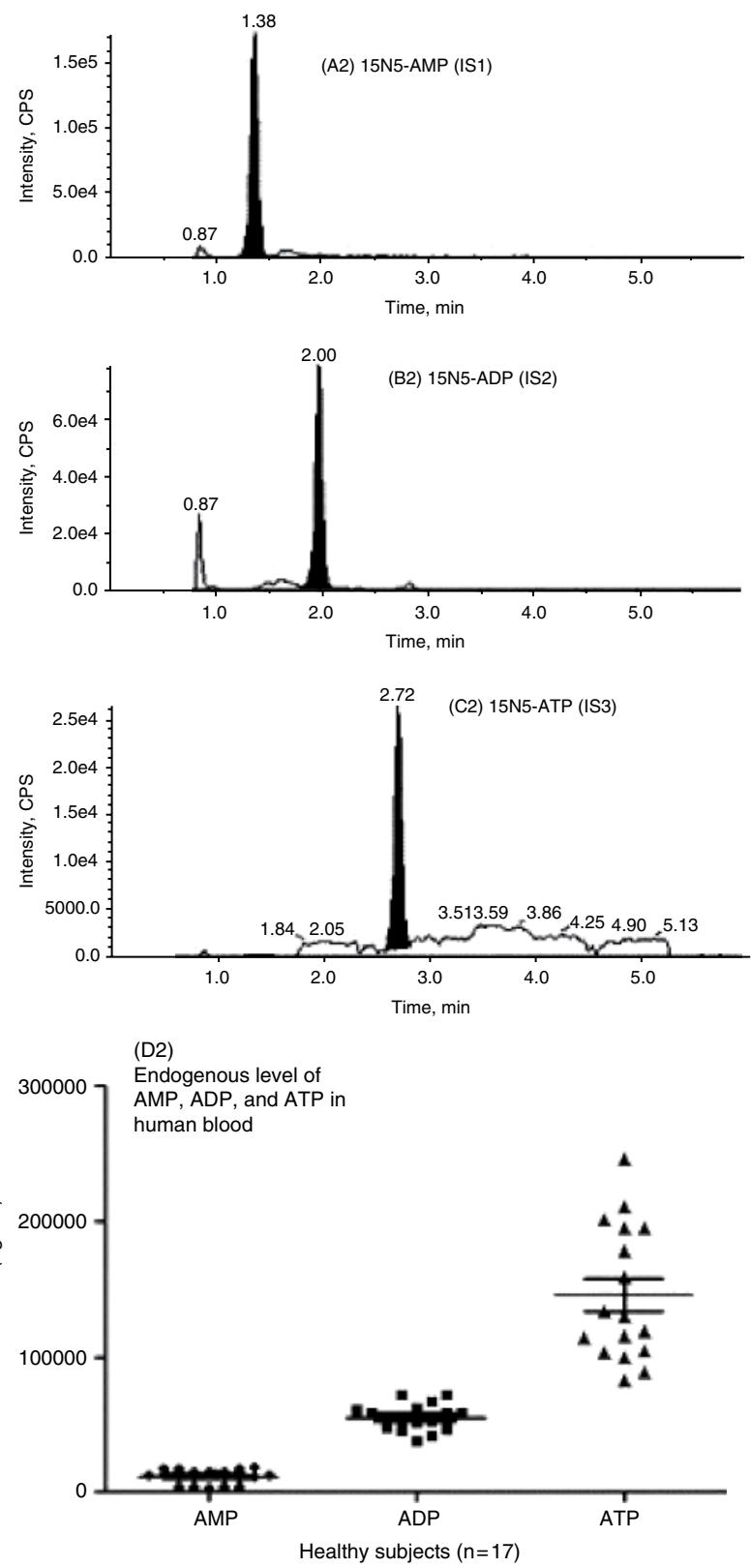
During the last decade, LC-MS/MS is becoming the key technique for nucleosides/nucleotides measurement in biological samples due to its excellent sensitivity, specificity, and accuracy.

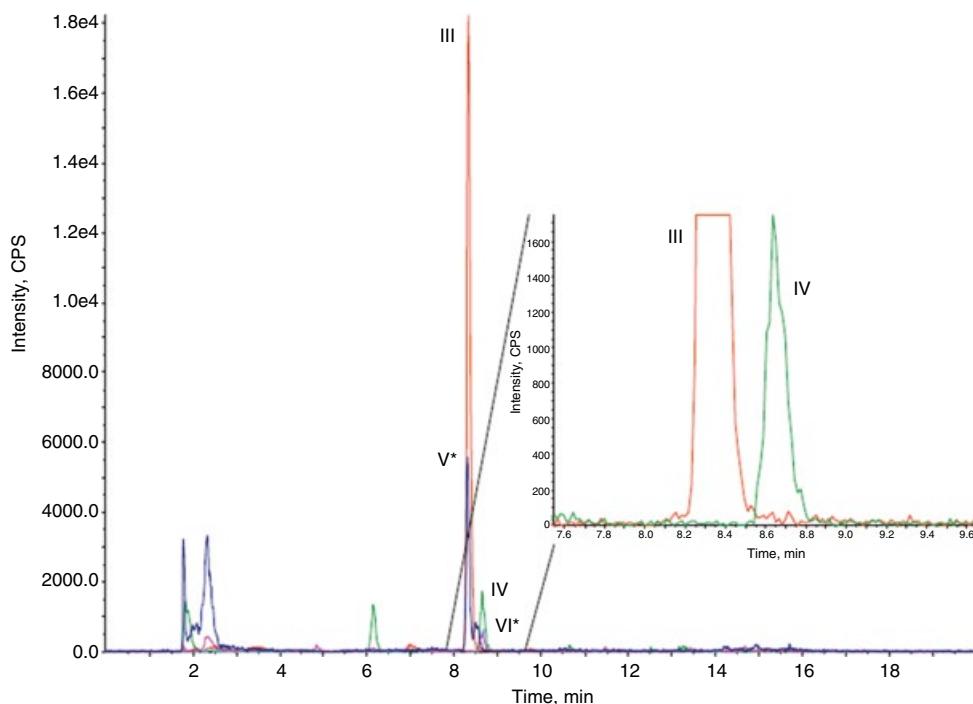
Nowadays, cutting-edge LC-MS/MS techniques are heading toward the most sensitive bioanalytical methods that enable quantitation of nucleoside/nucleotide biomarkers at very low concentrations in blood, plasma,

or tissues. LC-MS/MS also allows reproducible and efficient handling of large number of samples. This enables a clearer understanding of the role that nucleosides/nucleotides play in the emergence of certain diseases, which is important not only for diagnosis purposes in clinical labs but also for the research and development of new drugs. This chapter may be considered as an informative source for LC-MS/MS assays for measurement of nucleoside/nucleotide biomarkers in biological samples.



**Figure 25.3** Representative LC-MS/MS chromatograms of monitoring endogenous AMP, ADP and ATP in the commercial healthy human plasma ( $\text{K}_2\text{EDTA}$ ), (A1) 236.8 ng/mL of basal level of AMP in an individual human plasma, (A2)  $^{15}\text{N}_5\text{-AMP}$  (IS1), (B1) 119.1 ng/mL of basal level of ADP in an individual human plasma, (B2)  $^{15}\text{N}_5\text{-ADP}$  (IS2), (C1) 210 ng/mL of basal level of ATP in an individual human plasma, (C2)  $^{15}\text{N}_5\text{-ATP}$  (IS3), (D1) the endogenous level of AMP, ADP and ATP in individual healthy human plasma ( $n=15$ ) and (D2) The endogenous level of AMP, ADP, and ATP in individual healthy human blood ( $n=17$ ). Source: Zhang et al. (2014). Reproduced with permission of Elsevier.

**Figure 25.3 (Continued)**



**Figure 25.4** Representative MRM chromatogram of a human plasma sample after SPE on silica and analysis by LC-MS/MS. III. 3',5'-cAMP and IV. 3',5'-cGMP. Spiked with 5 ng/mL internal standard of  $^{13}\text{C}_{10},^{15}\text{N}_5$ -3',5'-cAMP (V\*) and  $^{2}\text{D}_1,^{15}\text{N}_3$ -3',5'-cGMP (VI\*). Measured concentrations are 6.4 and 0.6 ng/mL respectively for 3',5'-cAMP and 3',5'-cGMP. A zoom of the two quantifier daughter ions for 3',5'-cAMP and 3',5'-cGMP is also displayed. Source: Van Damme et al. (2012). Reproduced with permission of Elsevier.

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## 26

### LC–MS of RNA Biomarkers

Michael G. Bartlett<sup>1</sup>, Babak Basiri<sup>1</sup>, and Ning Li<sup>2</sup>

<sup>1</sup> Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, GA, USA

<sup>2</sup> Department of Pharmaceutical Analysis, School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, China

#### 26.1 Introduction

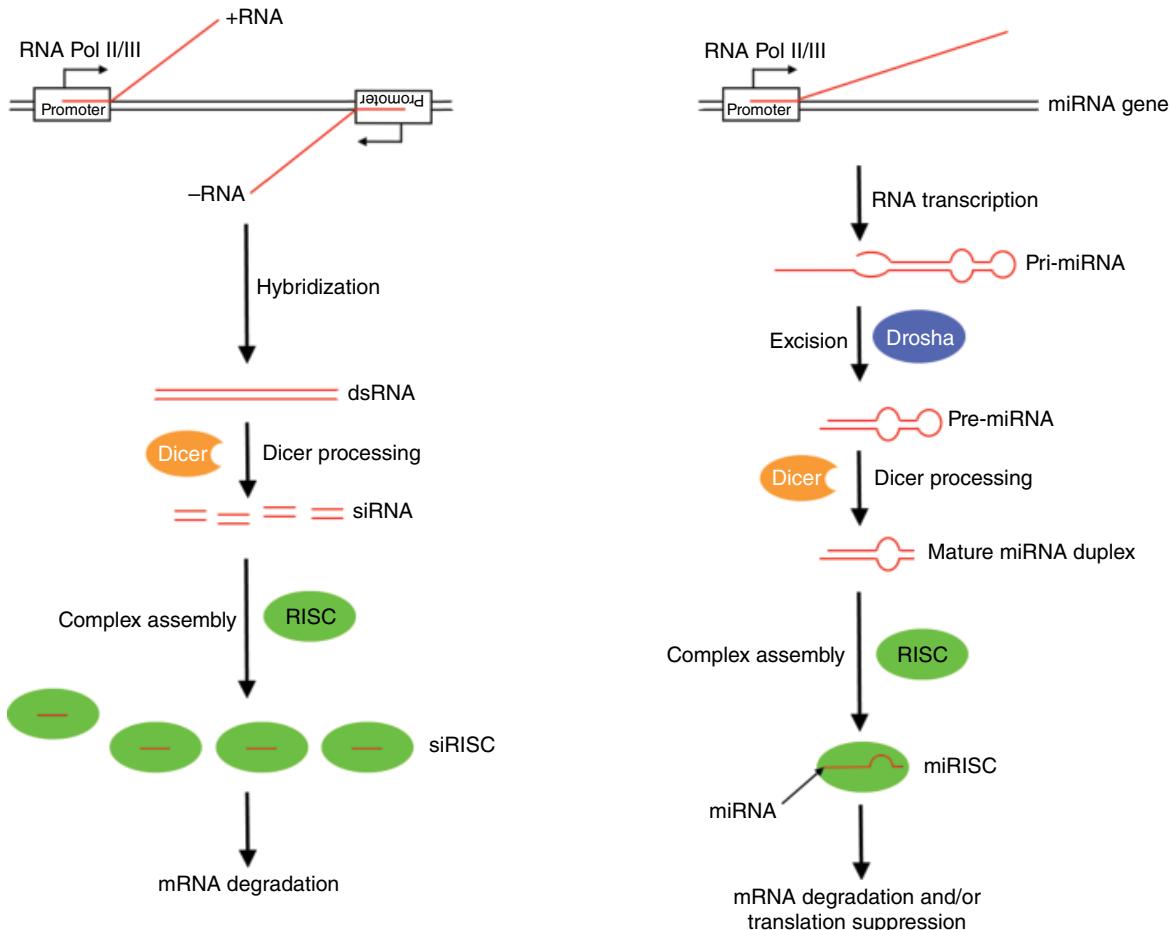
Recent updates from the Encyclopedia of DNA Elements (ENCODE) project indicate that approximately 80% of the human genome is transcribed (ENCODE Project Consortium, 2012). Yet only 1–2% of transcribed ribonucleic acids (RNAs) encode for proteins (Pennisi, 2012). The overwhelming majority of these transcription products are represented by noncoding RNA (ncRNA) (Washietl et al., 2007). It is becoming increasingly clear that many ncRNAs have significant biological roles (van Bakel et al., 2010). Therefore, a great deal of current research is aimed at understanding the physiological significance of this remaining, as of yet, undefined transcriptional activity.

Many of the biological roles of the noncoding regions of our genome are related to maintenance and regulation of the structure of chromosomes, such as telomeres and centromeres (Engreitz et al., 2013; van Wolfswinkel and Ketting, 2010). However, what has drawn the attention of so many are the noncoding regions that have demonstrated the ability to regulate the expression of protein-coding genes. This regulation has been found to have several distinct mechanisms such as altering mRNA translation (Jackson and Standart, 2007), altering chromatin structure (Magistri et al., 2012), methylation of DNA (Di Ruscio et al., 2013), and the regulation of other ncRNAs (Chen et al., 2012). The roles that these ncRNAs play in regulating mRNA translation has drawn the greatest attention and caused investigations into the potential of these molecules to be biomarkers.

Many categories of ncRNAs such as small nucleolar RNAs (snoRNA), long noncoding RNAs (lncRNA), long intervening noncoding RNAs (lincRNA), and guide RNAs (gRNA) have been previously described that can regulate the activity of other genes (Cech and Steitz, 2014). However, the majority of gene regulatory ncRNAs

can be placed in three general classes based on their mechanism of action (Appasani et al., 2009). The first two classes are formed by the protein dicer, which is responsible for cleaving larger RNAs into smaller fragments (Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). The major distinction between the two classes of dicer are that microRNAs (miRNAs) begin as larger single-stranded hairpin structures called pre-miRNAs while small interfering RNAs (siRNAs) are double stranded (Ying et al., 2009). In both cases, the protein dicer is able to produce small 20–24 nucleotide single-stranded molecules that are complementary to their target mRNA sequence (Elbashir et al., 2001a, 2001b; Flynt and Lai, 2008). These complementary sequences are directly incorporated into the RNA-induced silencing complex (RISC) where they form complexes with their target mRNA, initiating its degradation (Du and Zamore, 2005). The major elements of this pathway are shown in Figure 26.1.

The third major class is the piwi-interacting RNAs (piRNAs). They are slightly larger than miRNA and siRNA containing between 26 and 31 nucleotides (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006). A peculiar feature of piRNAs is that they are 2'-O-methylated at their 3' ends (Kirino and Mourelatos, 2007; Ohara et al., 2007). The mechanism that leads to their formation is not fully understood but does not involve dicer (Houwing et al., 2007; Vagin et al., 2006). However, they also cause the degradation of mRNA in a similar manner facilitated by RISC (Murota et al., 2014; Ohtani et al., 2013). The major functions of piRNAs are believed to be transposon silencing especially during embryo development in humans (Saito et al., 2006; Sarot et al., 2004). They also play a major role in spermatogenesis (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004).



**Figure 26.1** Biogenesis pathways for siRNA and miRNA.

## 26.2 Role in Disease and Therapeutics

There have been enormous efforts made to investigate correlations between disease state and changes in the expression of various ncRNAs (Hauser et al., 2012; Jacob et al., 2013; Kumar et al., 2013; Lorenzen and Thum, 2012; Mishra, 2014; Mitchell et al., 2008; Prensner et al., 2014; Qi and Du, 2013; Reis and Verjovski-Almeida, 2012; Schultz et al., 2014; Widera et al., 2011; Zhou et al., 2015). Interestingly, many of these ncRNAs are secreted from cells into extracellular fluids where they remain surprisingly stable (Gupta et al., 2010; Kosaka et al., 2010). This had led many to consider if bodily fluids such as plasma, urine, and even saliva may be capable of providing similar diagnostic capabilities as more invasive biopsy procedures. The term *liquid biopsy* is now being used to describe this concept (Karachaliou et al., 2015). The ability to take minimally invasive samples from patients and generate clinically relevant information is the ultimate goal of any biomarker study. In the case of

ncRNAs, there is growing evidence that this may become common practice in the near future.

Recent limited clinical studies have shown that ncRNAs are useful in predicting many diseases such as multiple sclerosis (Jr Ode et al., 2012), Alzheimer's disease (Kumar et al., 2013), cardiovascular dysfunction (Gupta et al., 2010), and amyotrophic lateral sclerosis (Toivonen et al., 2014). However, the greatest attention has been given to their potential role in the diagnosis and treatment of cancer (Kosaka et al., 2010; Mishra, 2014; Mitchell et al., 2008). Several ncRNAs have been shown to be correlated with the progression of many malignancies such as non-small cell lung cancer (Xu et al., 2015), colorectal cancer (Wang et al., 2015), prostate cancer (Prensner et al., 2014), and breast cancer (Guzman et al., 2015). In each case, changes in the concentrations of a small number of ncRNAs (generally 3 or 4) have been shown to have strong statistical significance when correlated against disease progression and even responsiveness to treatment. For example, two clinical trial cohorts identified that elevated levels of miR-320e were strongly

associated with poorer outcomes when treated with 5-fluorouracil (Perez-Carbonell et al., 2015). A recent study also found that plasma levels of miR-199a-5p were significantly decreased in triple-negative breast cancer patients when compared with other types of breast cancer and normal patients, suggesting that this miRNA may be a specific biomarker for this type of breast cancer (Shin et al., 2015). Another study similarly found that miR-133a was significantly decreased in patients with non-small cell lung cancer (Lan et al., 2015).

Another class of ncRNAs that have recently emerged as potential biomarkers are lncRNA (Qi and Du, 2013; Reis and Verjovski-Almeida, 2012; Zhou et al., 2015). As opposed to the siRNAs, miRNAs, and piRNAs, these generally contain greater than 200 nucleotides (Kung et al., 2013; Wilusz et al., 2009). A number of these larger ncRNAs are highly conserved and have shown altered expression profiles relative to normal cells in leukemia and colon and liver cancers (Calin et al., 2007). However, almost assuredly due to their size, there has not been any use of LC–MS to look at these products. Still, there may be opportunities for approaches like matrix-assisted laser desorption/ionization mass spectrometry (MS) (Kirpekar et al., 1999; Lecchi et al., 1995; Wu and McLuckey, 2004) or possibly the use of nucleases to release a smaller portion of the full-length oligonucleotide that could then be used as a surrogate molecule (Wambua et al., 2012) as potential methods to study these compounds.

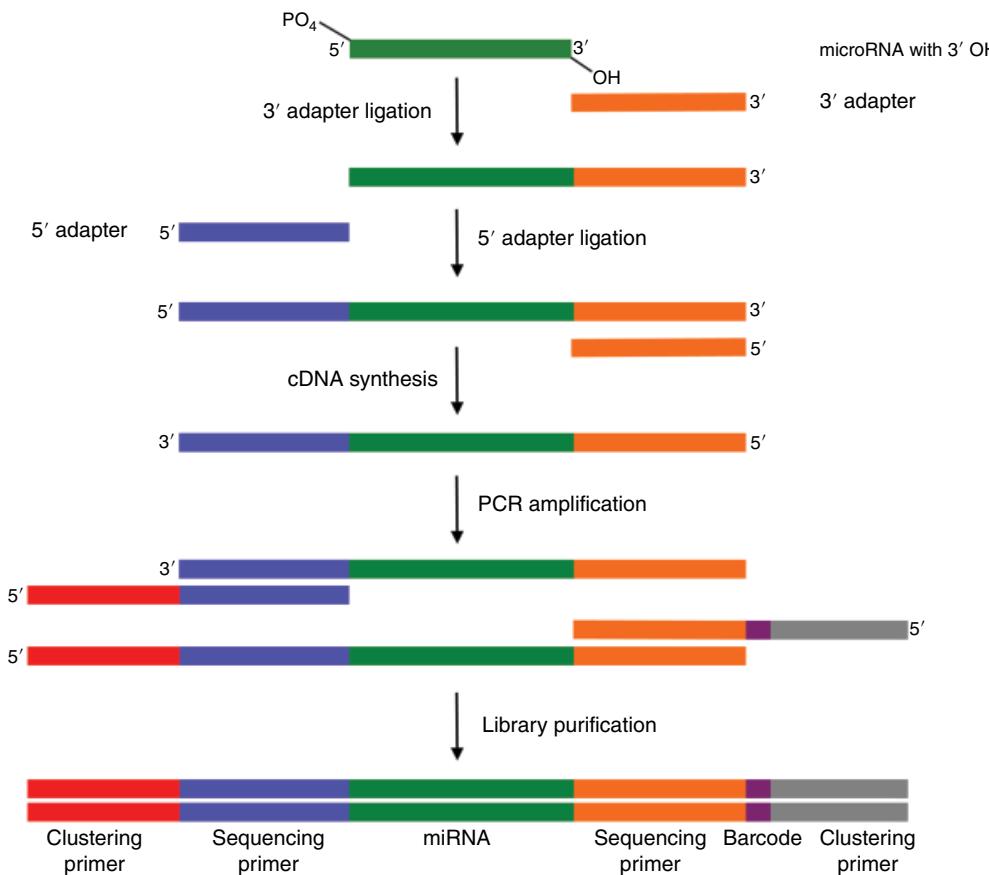
### 26.3 Role of Mass Spectrometry in RNA Biomarkers

Currently, for both basic and clinical studies, methods based on hybridization such as ELISA, different variations of qPCR, and microarrays are the gold standard for the analysis of oligonucleotides. However, without time-consuming and complicated adjustments to the protocol, these methods are not capable of distinguishing between the full-length and truncated or unmodified and modified versions of oligonucleotides, making them less likely to differentiate between a therapeutic and a closely related endogenous oligonucleotide (Chen et al., 2005; Cheng et al., 2009), and therefore they are not suitable for quality control or metabolic studies. They also demonstrate specific disadvantages for biomarker discovery due to problems with specificity, normalization, and biased performance of different PCR techniques (Wang et al., 2012, 2013). It has been reported previously that microarray-based miRNA detection methods cannot reliably distinguish between miRNAs that have one or only a few mismatches (Miska et al., 2004). And even though real-time reverse transcription polymerase chain

reaction (RT-PCR) is often considered as the ultimate standard for miRNA assays (Git et al., 2010), the use of miRNA qPCR panels from different commercial suppliers can provide inconsistent results. For example, in one study, plasma and serum miR-107 showed much higher levels when measured with Exiqon compared with TaqMan qPCR kits (Wang et al., 2012).

More recently the use of cDNA libraries followed by next-generation (Next-Gen) sequencing has been widely applied to the detection of mRNAs (Cobos Jiménez et al., 2014; Eminaga et al., 2001; Lu et al., 2007; Motamony et al., 2010; Tam et al., 2014). The quality of Next-Gen sequencing is completely dependent on the cDNA library, which must contain the miRNA that is being evaluated. However, library construction for Next-Gen sequencing of the highly complex and dynamic ncRNA pool of an organism can often introduce undesired bias (Git et al., 2010; Head et al., 2014; Lu et al., 2007). It is more difficult to detect base extensions of an miRNA unless such modifications have been adequately represented in the library. These techniques also depend, like Sanger sequencing, on some sort of PCR (emulsion PCR or bridge PCR) for the initial clonal amplification and library preparation (see Figure 26.2). As a result, they cannot sequence chemically modified DNA bases (e.g., methylcytidine) and rely on techniques such as bisulfite sequencing for characterization of epigenetic modifications (Morozova and Marra, 2008). Finally, the per-sample cost of Next-Gen sequencing is quite high (between \$1200 and \$2200 per sample) and requires 16–24 h to run a sample. It also requires a significant bioinformatics infrastructure to support the interpretation of the results. During the early phase discovery of miRNA biomarkers, the ability to look at hundreds of miRNAs in a single run is invaluable. However, once the tentative biomarker(s) has been identified, validation and subsequent analysis tasks should be transferred to techniques like RT-PCR and LC–MS that are both more economical and optimized for the analysis of a single or handful of analytes.

Direct measurement of these oligonucleotides appears to be even more imperative with the recent report of multiple modified species present for two different high concentration miRNA species that were interrogated by LC–MS (Katoh et al., 2009; Yu and Chen, 2010). This opens up the possibility that there is even greater complexity in miRNA regulation based on alterations in the concentrations of these multiple forms. For example, Abe et al. have demonstrated an age-associated increase in 2'-O-methylation of miRNAs in *Drosophila* with an indispensable impact on aging processes (Abe et al., 2014). In this study, mutations in the *hen1* gene, which is responsible for 2'-O-methylation of small RNAs, resulted in accelerated neurodegeneration and shorter life span.



**Figure 26.2** miRNA cDNA library construction for next-generation sequencing.

Clearly, there is a need for a technique that can directly measure and quantitate these chemically modified species.

Previous successful MS-based measurements of other types of oligonucleotides in biological matrices (Beverly et al., 2006; Chen and Bartlett, 2013; Deng et al., 2010; Hemsley et al., 2012; Johnson et al., 2005a; Zou et al., 2008) as well as recent reports of the MS analysis of synthetic miRNA mixtures (Izumi et al., 2012; Kullolli et al., 2014) provide reasonable precedence to believe that LC-MS-based analysis of miRNAs from biological samples could become routine. Compared with qPCR techniques, MS can generate values that are more quantitative, easier to normalize, and less prone to bias (Wang et al., 2013). Moreover, native nucleic acid molecules usually undergo chemical modifications that are of functional biological importance (Karijolich et al., 2010). Therefore, the reliable identification of miRNA modifications with high confidence is of great importance in elucidating both their biological function and kinetic profile. Despite their superior sensitivity and relative ease of use, hybridization-based methods have difficulty in detecting post-transcriptional modifications such as methylation as well as truncations. Therefore, MS-based techniques have gained increasing importance in the identification of

modified small RNAs. The full scan mass (MS) spectra of RNAs, as well as multistep MS<sup>n</sup> analyses, have been successfully applied to identify sequence placement of modified RNAs (Farand and Gosselin, 2009; Hossain and Limbach, 2007; Lin et al., 2007). There are only a few direct MS analyses of an endogenous miRNA reported so far. One is of the highly expressed liver-specific miR-122 extracted from human hepatocytes and mouse livers. The acquired mass spectra revealed that one variant of miR-122 has a 3'-terminal adenosine that is introduced after processing by dicer (Katoh et al., 2009). A recent study by Nakayama et al. (2015) has identified 20 miRNAs from HeLa cells using LC-MS. Interestingly, several of them appear in multiple forms such as miR-23a, which has variants missing the 3'-cytosine and a variant with an additional 3'-adenosine. miR-30c had a variant with an additional 3'-uridine, and miR-27a had a variant that was also missing the 3'-cytosine. It has also been reported that *Arabidopsis* miR-173 has a 2'-O-methylation on its 3'-terminal ribose (Yu and Chen, 2010). This modification has been observed in *Drosophila* as well (Abe et al., 2014). Therefore, MS characterization of other miRNAs, especially extracellular miRNAs, is expected to provide valuable information regarding their

potential posttranscriptional modifications that are not readily obtainable by alternative methods. As demonstrated by the previously mentioned case of aging in *Drosophila* (Abe et al., 2014), such modifications can have important biological consequences.

## 26.4 LC-MS Approaches for RNA Determination

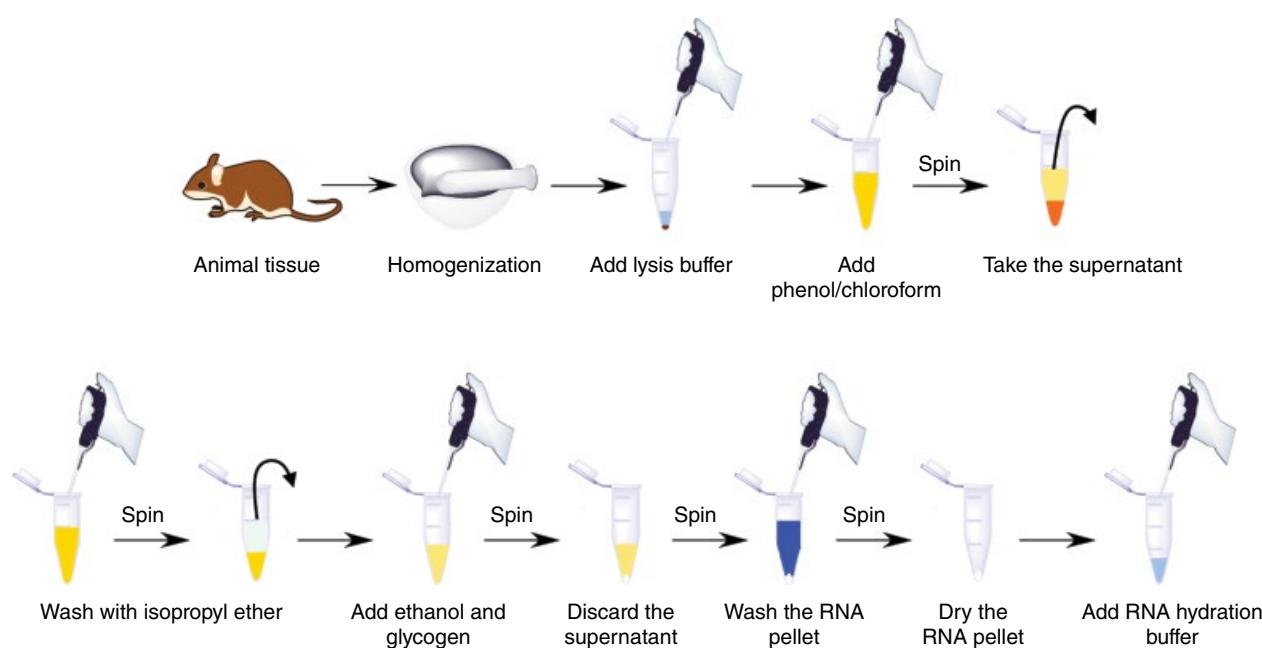
### 26.4.1 Sample Preparation

Bioanalytical methods begin with sample extraction from a biological matrix. The goal of sample preparation is to isolate the oligonucleotide from interfering compounds and to maximize the recovery of the analyte. There are five basic approaches for the isolation of oligonucleotides from the biological samples. It should be noted that because of nonspecific binding of analytes to storage containers (Deng et al., 2010; Zhang et al., 2007), all stock solutions, work solutions, and samples should be prepared in containers that have been designed to minimize binding to oligonucleotides. The first method is a classic liquid–liquid extraction (LLE) using phenol/chloroform (generally in a 2:1 ratio) (Beverly et al., 2006; Griffey et al., 1997; Murphy et al., 2005). This extraction method is improved when the pH of the samples is initially increased using a base like ammonium hydroxide. The mixture is simply vortexed and centrifuged. The aqueous layer is transferred next to a new tube and washed with isopropyl ether to remove other endogenous compounds.

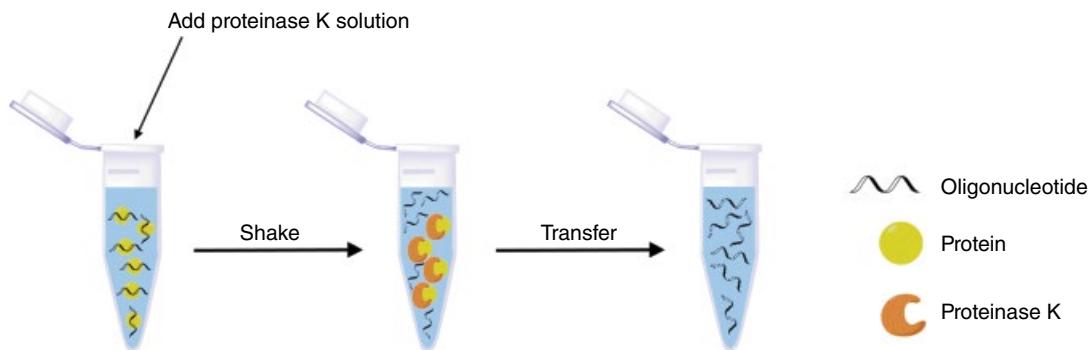
The aqueous phase is mixed with a glycogen solution and ethanol before storage at -80°C for precipitation. Glycogen acts as an inert carrier and is widely used to increase the recovery of RNA during alcohol precipitations. Once the precipitate forms, the solution will be centrifuged at 0°C. The supernatant will be removed and the precipitated RNA will be air-dried in a fume hood. A schematic of this process is found in Figure 26.3. The final sample is then reconstituted in the initial chromatographic mobile phase prior to injection into the LC-MS system.

The second approach involves using proteinase K to enzymatically remove the proteins from samples leaving the oligonucleotides behind (Bellon et al., 2000; Bourque and Cohen, 1993; Chen et al., 1997; Raynaud et al., 1997; Shimizu et al., 2012). In this method the biological sample is buffered using tris(hydroxymethyl)aminomethane (Tris). Several other additives are needed to improve this digestion such as dithiothreitol (DTT) to reduce disulfide bonds in the proteins and the chaotropic agent guanidinium chloride. Ethylenediaminetetraacetic acid (EDTA) is also added to sequester metal atoms that are critical cofactors in several ribonucleases and thereby protect the oligonucleotides during digestion. Finally the enzyme proteinase K is added and the sample is digested for several hours while shaking. After digestion, the sample will be centrifuged and the supernatant taken for LC-MS analysis (McGinnis et al., 2013a). A schematic of this process is shown in Figure 26.4.

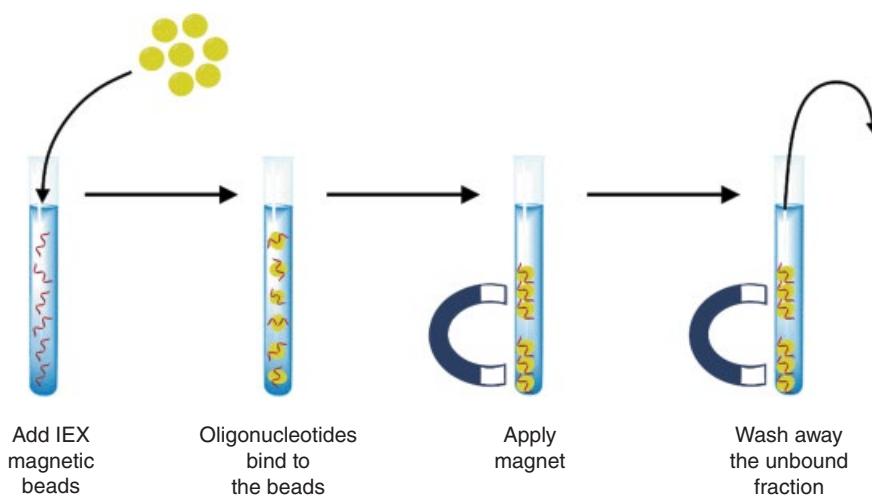
The third approach involves the use of magnetic ion exchange beads (Ye and Beverly, 2011). In this case, a



**Figure 26.3** Schematic representation of phenol/chloroform LLE.



**Figure 26.4** Proteinase K digestion for oligonucleotide extraction.



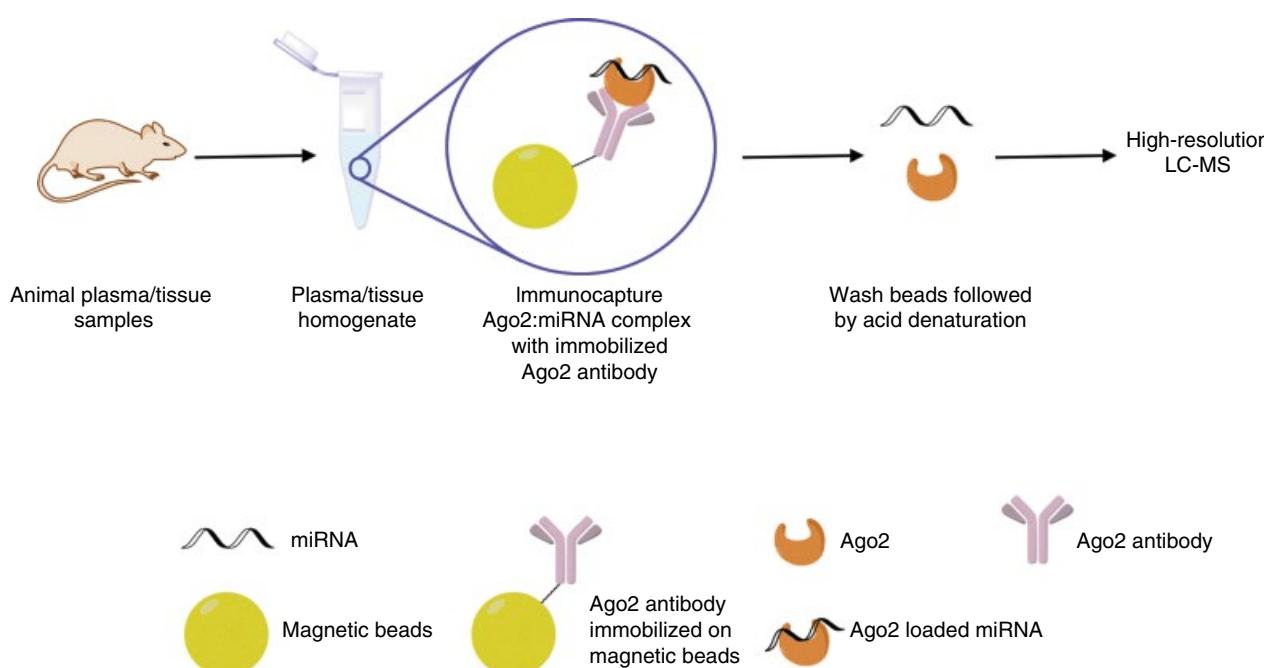
**Figure 26.5** Schematic representation of the ion exchange beads for oligonucleotide sample preparation.

plasma sample with added internal standard is brought up to volume with slightly acidic water. The diluted plasma sample is mixed with the beads by agitation while pipetting. The samples are then placed in a magnetic ring to separate the beads, allowing removal of the supernatant. The beads are washed with an acidic solution containing a small amount of the surfactant polyoxyethylenesorbitan monolaurate (Tween). The oligonucleotide is then eluted from the beads by using a high concentration solution of ammonium chloride. Samples need to be filtered prior to injection into an LC–MS system to ensure removal of any residual magnetic beads. Figure 26.5 shows a schematic of this extraction process.

The fourth approach involves combining magnetic beads with the immunocapture of RISC-loaded RNAs using Argonaute 2 (Ago2) monoclonal antibodies (Zarovni et al., 2015). In this approach shown in Figure 26.6, protein G magnetic beads are used to capture either anti-mouse Ago2 (for mouse or rat samples) or antihuman Ago2 (for monkey or human samples). The magnetic beads are maintained at physiological conditions using a solution of Tris and sodium chloride. The beads are incubated overnight with the appropriate

anti-Ago protein. Plasma samples are added to the beads along with EDTA to scavenge metal cofactors needed for RNases and a lysis buffer (NP40) in nanofiltered water. The miRNAs are released from the magnetic beads using acetic acid. The samples are then neutralized with ammonium hydroxide and buffered using Tris and triethylammonium acetate (TEAA) prior to analysis using LC–MS.

A fifth approach involves the use of solid-phase extraction (SPE) for the isolation of RNA (Dai et al., 2005; Johnson et al., 2005a). In this approach three buffers are needed. An equilibration buffer is composed of ammonium acetate at pH 5.5. A washing buffer is made by mixing equal parts of the equilibration buffer with acetonitrile. Finally, the elution buffer is made by dissolving ammonium bicarbonate with a small amount of tris-(2-carboxyethyl)phosphine (TCEP) in water and adjusting the pH with ammonium hydroxide to 9.5 (Chen and Bartlett, 2012). The use of TCEP dramatically reduces oxidation during sample preparation but may not be necessary for all samples depending on their inherent sensitivity to air. This buffer is then mixed with equal parts of a 4:1 mixture of acetonitrile and THF.



**Figure 26.6** Immunocapture magnetic beads.

The SPE cartridges are conditioned using methanol and the equilibration buffer. Plasma samples were then mixed with a lysis buffer (either Clarity OTX buffer or a buffer similar to the one described in approach 2) (Tris, DTT, guanidinium chloride, and EDTA) and loaded onto the column. Some lower molecular weight compounds are removed using the washing buffer and then the analytes are collected using the elution buffer. The collected solutions are evaporated to near dryness under vacuum and reconstituted in deionized water prior to LC-MS analysis.

These methods can also be used in combination with each other. For example, LLE has been used in combination with SPE, which has proven to be highly effective (Deng et al., 2010; Zhang et al., 2007). More recently, using a combination of phenol/chloroform LLE and ethanol precipitation, Chen and Bartlett managed to achieve recoveries of more than 75% while analyzing a phosphorothioate DNA oligonucleotide (Chen and Bartlett, 2013).

All of these approaches have been successfully used for at least one class of oligonucleotides, but none of these approaches have been evaluated broadly. The analytical figures of merit for each approach are summarized in Table 26.1. In many cases, the recovery for the oligonucleotide is at least 70%. In addition, each process has been used to prepare samples that are compatible with LC-MS analysis of the isolated oligonucleotide. One final approach that should be mentioned is fractionation of samples. Since endogenous RNAs exist over widely differing sizes, isolating the smaller RNAs that comprise the

biomarkers has merit. There are two basic approaches that could be used. The first is to use porous columns to collect the various chromatographic fractions (Kim et al., 2007; Yamauchi et al., 2013). This approach has been used to look at cell culture samples. The second approach would be to use centrifugation to enrich samples. This approach would be ideal for collecting extracellular exosomes, which are known to contain high levels of small RNA molecules (Greening et al., 2015). However, it is not clear if using these types of approaches for plasma or tissue samples alone or prior to employing one of these other approaches would improve assay ruggedness.

#### 26.4.2 Ion-Pair Chromatography

Oligonucleotides have several significant chromatographic challenges, such as their highly polar nature and their charged backbone. These two key physiochemical features are the major contributors to the need for ion-pairing agents in the LC-MS analysis of oligonucleotides. Ion-pairing agents are amphipathic molecules possessing a charged head group on one end and a hydrophobic tail on the other (Bartha and Ståhlberg, 1994). The charged head groups form ion pairs with the phosphate backbones of the oligonucleotides, increasing their interaction with the stationary phase in the column (van Dongen and Niessen, 2011). However equally important, the transient ion pairs increase the transport of oligonucleotides to the surface of electrospray droplets and assist in their transition into the gas phase (Chen

**Table 26.1** Comparison of oligonucleotide recovery values using various sample preparation methods.

Extraction method	Analyte recovery	Reference
LLE	30–70%	Zhang et al. (2007)
	70–79%	Deng et al. (2010)
	75 ± 14%	Bergallo et al. (2006)
SPE	23–80%	Johnson et al. (2005)
	30–64%	Dai et al. (2005)
	60–80%	Chen and Bartlett (2012)
Magnetic IEX beads	64–99%	Ye and Beverly (2011)
Proteinase K treatment	≈50%	Chen et al. (1997)
	98 ± 3%	Raynaud et al. (1997)
	≥95%	McGinnis et al. (2013a)

et al., 2013). It is the combination of improving both chromatographic performance and electrospray mass spectral response that has made this approach so widely used.

Over the past 20 years, LC-MS of various oligonucleotides has mostly been accomplished using a mobile phase consisting of an aqueous buffer of triethylamine (TEA) and hexafluoroisopropanol (HFIP) with an organic phase consisting of methanol (Apffel et al., 1997a, 1997b). However, recently several combinations of alternative alkylamines with HFIP have been shown to provide improved performance for selected groups of oligonucleotides (Bartlett et al., 2013; Basiri and Bartlett, 2014). The ion-pairing agent butyldimethylammonium bicarbonate has been shown to increase the retention of oligonucleotides and thus allow for higher organic content in the mobile phase at the time of elution, resulting in increased electrospray sensitivity (Oberacher et al., 2001a, 2001b). Another extensive study by Chen et al. showed a dramatic improvement in the LC-MS performance for phosphorothioate oligonucleotides when using the ion-pairing agent diisopropylethylamine (Chen and Bartlett, 2013). One of the more interesting recent studies was by McGinnis et al. (2013b) who looked at the sense and antisense strands of an siRNA and found that the two strands showed optimal response with different ion-pairing agents. Finally a recent study by Oberacher showed that certain ion-pairing agents can dramatically reduce the amount of alkyl metal adduction observed in the electrospray mass spectra of oligonucleotides (Erb and Oberacher, 2014). This has important implications when trying to determine the identity of unknown compounds such as synthetic impurities or metabolic products. One of the major findings from these studies is that

optimizing the mobile phase composition can dramatically improve the sensitivity of LC-MS methods. It is also clear that the interaction between ion-pairing agents and oligonucleotides during LC-MS is critically important to the success of the analysis but is still not completely understood. Currently there are several mobile phase compositions that appear to provide acceptable performance for analytes depending on the hydrophobicity of the oligonucleotide. The following suggestions can be considered guidelines based on the current state of the literature (McGinnis et al., 2013b). For more hydrophilic oligonucleotides such as miRNA or siRNA, a buffer containing diisopropylamine (DIPA) with hexafluoroisopropanol should be used. Tripropylamine with hexafluoroisopropanol has reasonable performance for unmodified DNAs. Finally, diisopropylethylamine with hexafluoroisopropanol appears to work well for more hydrophobic phosphorothioate-containing oligonucleotides. In all cases ethanol is suggested to be the organic component of the mobile phase (Chen and Bartlett, 2013).

Despite not having a full understanding of the interactions between ion-pairing agents and oligonucleotides, there have been great advances toward recognizing the fundamental physicochemical forces involved in these interactions. It is clear that the Henry's law constant of the ion-pairing agent, which describes the partition coefficient between the solution and gas phase, has a strong correlation with the electrospray ionization response of an oligonucleotide. It also correlates with the observed charge state distribution of the electrospray ionization envelope. These correlations have implications into the mechanism of electrospray desorption. Chen et al. (2013) have proposed a model that suggests that ideal ion-pairing agents will concentrate in the electrospray droplet following preferential loss of hexafluoroisopropanol from the electrospray droplet. This loss leads to ionization of oligonucleotides via a mechanism known as "wrong-way-round ionization." However, as the study by McGinnis et al. (2013b) clearly demonstrates, the interaction cannot be fully explained by any single physicochemical factor and in reality likely involves both the properties of the analyte and mobile phase.

### 26.4.3 Capillary Chromatography

The use of capillary chromatography generally improves method sensitivity relative to conventional chromatographic approaches. Therefore, it is not surprising that these approaches have been used with oligonucleotides. Some of the most impressive work has been conducted by Oberacher et al. who have employed monolithic capillary columns (Hölzl et al., 2004; Huber et al., 2001; Premstaller et al., 2000). The use of monolithic columns eliminates the most significant drawback with using

capillary columns, the long run times due to poorer mass transfer. In this case, they have baseline separated polyT ladders ( $T_{12}$  through  $T_{18}$ ) in less than 6 min at flow rates of  $3\text{ }\mu\text{L/min}$ . This group also has developed an interesting sample preparation technique. They recommend the addition of a 900-fold molar excess of EDTA to the sample prior to injection. The EDTA was chromatographically resolved from the oligonucleotide, but its presence in the injection solution reduced the cationic adduction that is generally observed in the electrospray ionization mass spectra of oligonucleotides (Oberacher et al., 2004). This approach has been used with oligonucleotides ranging in size from 21 to 55 nucleotides up to an intact 5S rRNA subunit.

Another noteworthy use of capillary chromatography for the analysis of small RNAs was conducted by Dickman and Hornby (2006). In this study, miRNAs from a total RNA extract of HeLa cells was separated. The study was specifically targeting the Let-7 miRNA, and they found that they could separate this individual miRNA from the total RNA extract. This study is particularly noteworthy because this miRNA has been shown to regulate RAS expression in humans (Johnson et al., 2005b, 2007). The expression of Let-7 is low when the expression of RAS is high in cancerous cells, while Let-7 expression is high and RAS levels are low in normal cells. Therefore, Let-7 is an attractive potential therapeutic agent. Toward this goal, it has been shown that intranasal delivery of Let-7 is effective in slowing tumor growth in a mouse model of lung cancer (Trang et al., 2010).

#### **26.4.4 Liquid Chromatography-Inductively Coupled Plasma Mass Spectrometry**

The use of inductively coupled plasma mass spectrometry (ICP-MS) as a detector for MS has some interesting features. Detection occurs via the phosphorus oxide ion ( $m/z$  47), which is formed following combustion of the oligonucleotide after eluting the column. The initial studies involved the use of hydrophilic interaction liquid chromatography (LC) for the separation of oligonucleotides. However, the authors were only capable of providing baseline separation between the full-length oligonucleotide and n-5 sequences (Easter et al., 2010). A more recent study by Buszewski and coworkers has shown that ICP-MS can be used with ion-pair LC separations using the TEA/HFIP buffering system (Studzinska et al., 2015). They did find that the concentrations of TEA and HFIP directly influenced method background and therefore should be minimized. They were able to demonstrate their method for the quantitative determination of a phosphorothioate-containing oligonucleotide and its metabolites from serum. Liquid chromatography-inductively coupled plasma mass

spectrometry (LC-ICP-MS) to date has shown promise as a detection method for quantitating oligonucleotides. However, since the signal is less specific than methods that detect the intact molecular species, there needs to be significant effort applied to the quality of the chromatographic separation.

## **26.5 Case Studies**

### **26.5.1 Single Nucleotide Polymorphisms as Biomarkers**

Single nucleotide polymorphisms (SNPs) are DNA variations occurring commonly within a population. The genomic distribution of SNPs in humans is not homogeneous. In addition, the frequency of SNPs varies between human populations such that an allele that is common in one region or ethnic group may be quite rare in another. These genetic variations have been shown to predict the severity of disease and responsiveness to treatments (Shastry, 2007). The classic example is the E4 SNP in the gene coding for apolipoprotein E. This SNP has been correlated with increased risk for Alzheimer's disease (Mahley et al., 2006).

LC-MS analysis for SNPs has been conducted following PCR-amplified fragments of intracellular nucleic acids. The oligonucleotides can be subjected to MS/MS sequencing after an initial chromatographic separation for confirmation. It has been shown that precursor ions with higher charge states generate non-sequence-specific fragments at low  $m/z$  values, while lower charge state precursor ions give a greater number of high  $m/z$  sequence-specific fragments (Deng et al., 2010; Little et al., 1995, 1996; McGinnis et al., 2012). As discussed earlier, ion-pairing agents with extremely low or high Henry's law values have been shown to reduce oligonucleotide charge state distributions (Chen et al., 2013). Certain ion-pairing agents also reduce alkali metal ion adduction due to their interaction with the polyanionic backbone of the oligonucleotides. These ion-pairing agents can displace metal cations and then later dissociate from the oligonucleotide backbone during the final stages of ionization. Another effective means of reducing the charge state of oligonucleotides is through the use of proton transfer reactions. In this case, protonated ions such as benzoquinoline can be reacted with oligonucleotides, decreasing their charge state (Wu and McLuckey, 2003). Other factors that have been shown to reduce adduct formation in electrospray ionization are increased column temperatures and the addition of metal chelators like EDTA during sample preparation. Elevated column temperature employs two mechanisms to reduce metal ion adduction. First, it improves the association of

ion-pairing agents, relative to alkali metals, with the phosphate backbone. Second, it facilitates the formation of single-stranded DNA fragments with little to no secondary structure. Forming single-stranded oligonucleotides is important because it has been shown that the desalting of single-stranded oligonucleotides is easier as the binding energy of alkali metals is lower for single-stranded than double-stranded oligonucleotides (Beverly et al., 2005).

Capillary ion-pair reversed-phase HPLC combined with ESI-MS has been used to identify polymorphic short tandem repeat loci from PCR-amplified fragments of the human tyrosine hydroxylase (*humTH01*) gene (Oberacher et al., 2001b). In this example, allele 9.3 is a common variant of allele 10 that has a deoxyadenosine deletion. The two alleles were readily identified based on their masses that differed by only a single nucleobase (6 Da mass shift from a total mass of 31,000).

### 26.5.2 Small Interfering RNA Determination

Small interfering RNAs are endogenous regulators of protein expression that have been used as powerful tools to validate biochemical pathways and therapeutic targets. There have been several studies that have investigated this class of ncRNAs. One particular LC-MS study investigated the metabolism of an siRNA compound that was targeted against the enzyme-inducible phospholipase A2 $\beta$  (iPLA2 $\beta$ ) (McGinnis et al., 2013b). This study used ion-pair reversed-phase chromatography. Twelve alkylamines were tested with respect to the electrospray ionization signal intensity with both sense and antisense strands of the siRNA. The four most promising ion-pairing agents were selected, and the HFIP concentration and chromatographic separation were optimized while minimizing ion suppression from co-eluting analytes. DIPA was selected as the final ion-pairing agent. Sample preparation used a modified chloroform/phenol extraction to enrich samples for short RNAs. Using LC-MS, four metabolites were identified: the siRNA 5'-(N-1) and 5'-(N-2) from the antisense strand and 5'-(N-1) and 5'-(N-2) from the sense strand. The identification of the metabolites was further confirmed by synthesizing the sequences. The use of the extraction resulted in molecular signals that were primarily potassium adducts as well as sodium adducts of both full-length single strands and metabolites. There was no inclusion of a desalting step that could have reduced or altered the addition with potassium and other salts resulting from the TRIzol procedure and would likely facilitate the identification of unknowns.

As further verification of the metabolism of this siRNA, these authors examined the base composition of the 5' sense and antisense strands. The A:U base pair

contains two hydrogen bonds, while the C:G base pair contains three hydrogen bonds. The 5' end of the antisense strand contains the sequence UGA, while the 5' end of the sense strand contains GGA. This indicates that the 5' end of the antisense strand is less thermodynamically stable than the 5' end of the sense strand (7 vs. 8 hydrogen bonds). Thus exonuclease activity would be expected to be greater at this end, which is consistent with the observations in this study. The authors also noted that metabolism occurred principally through exonucleases. These findings agreed with the earlier work of Beverly and coworkers (2005, 2006) as well as work from Shimizu et al. (2012).

### 26.5.3 MicroRNA Determination

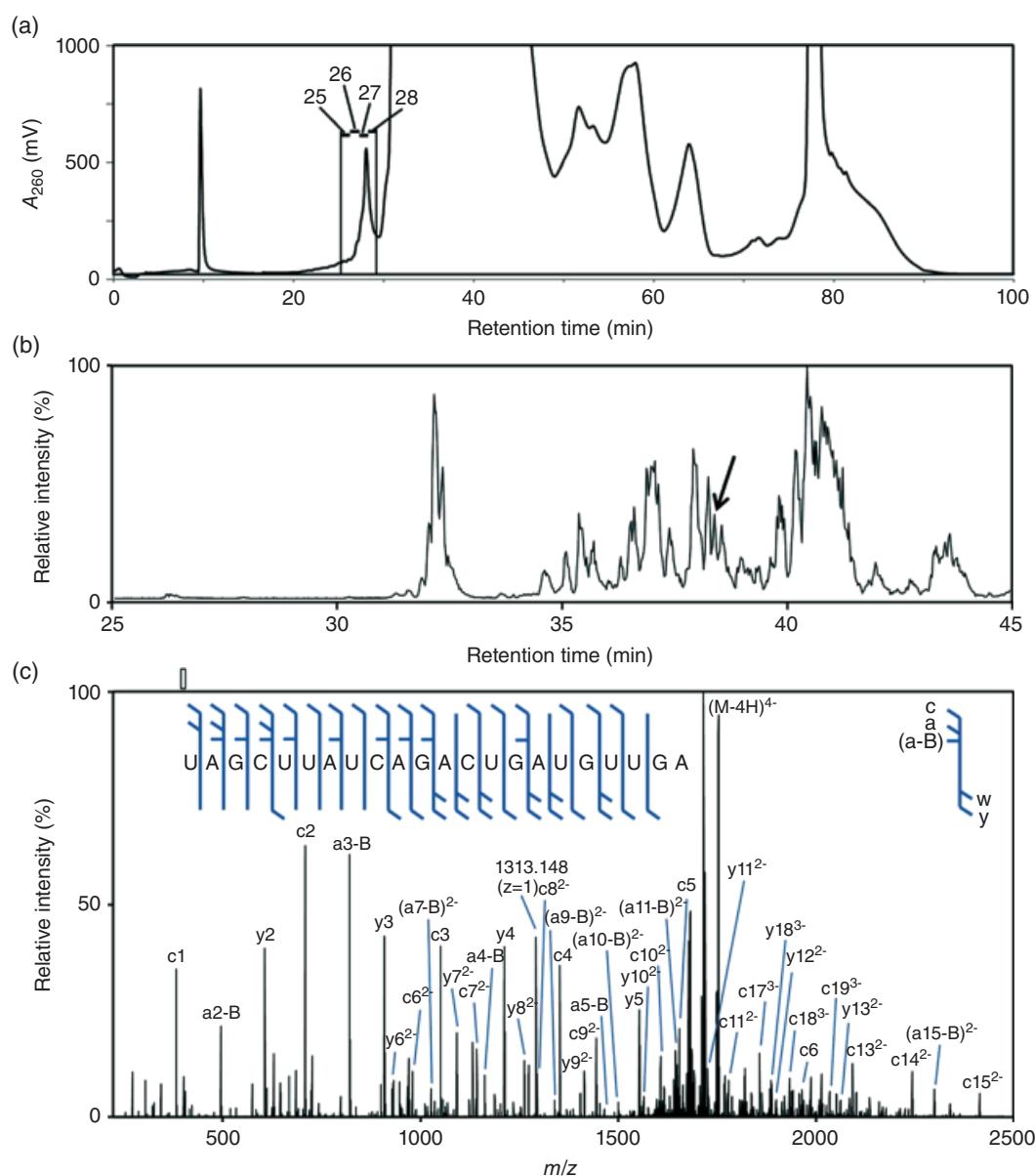
MiRNAs comprise one of the largest classes of ncRNAs. Currently, more than 1800 miRNAs have been found in the human genome. Since approximately 60% of protein-encoding genes are regulated in some fashion by miRNAs, there is little surprise that there has been enormous recent interest in this class of biomolecules. Recent work by Isobe and coworkers has transformed the field of LC-MS of miRNAs (Nakayama et al., 2015). The development of an ion-pairing reversed-phase LC method employing a macroporous polystyrene–divinylbenzene resin allows for fractionation of complex biological samples into RNAs ranging in size from 20 to 8000 bases in length (Yamauchi et al., 2013). Nonporous and monolithic versions of this resin have been previously used with RNAs, but they were found to have poor retention and separation capabilities for larger RNA molecules. Therefore, those columns should be primarily used for oligonucleotides below 1000 bases in length.

The strategy was further refined by using a small RNA isolation kit on HeLa cells (Nakayama et al., 2015). Next a 2 × 100 mm macroporous polystyrene–divinylbenzene column was used to purify the small RNAs prior to injection into a nano-LC system for LC-MS/MS analysis. The nano-LC system employed a C18 trapping column to remove low molecular weight compounds prior to separation on a C30-UG3 resin. The authors found that when the organic content of the mobile phase was below 20–30% (depending on the organic solvent used), it was necessary to employ an orthogonal nanospray emitter with 100% organic solvent to stabilize the spray emanating from the LC. For mass spectral detection, they used an Orbitrap mass analyzer that was operated using a data-dependent scanning function that would allow for the collection of MS/MS data on peaks as they eluted. The identification of miRNA species was conducted using database searching using the Ariadne algorithm, which calculated the mass values for miRNAs found in the human miRBase. One powerful feature of this

approach is that it considered not only the mature miRNAs but also 3' and 5' variants for a total of 55,176 possible sequences.

Using this approach they were able to identify 23 miRNA species from the HeLa cell extract as shown in Figure 26.7. This represents the first time that LC-MS has been applied in a nontargeted manner for the determination of miRNAs. The result of this approach is

the finding that five of these miRNA species existed in multiple forms. For example, miR-21 was identified with an additional C at the 3' end following full sequencing by MS/MS. Similar observations were made for miR-23a, which was found in three forms (the mature form and two modified forms), and miR-27a and miR-30c, which were only found in modified forms. Figure 26.7c shows also an excellent example of the



**Figure 26.7** Direct identification of a human cellular miRNA. (a) Separation of the small RNA fraction from HeLa cells by reversed-phase LC through a polystyrene–divinylbenzene column. (b) Base peak mass chromatogram of fraction 27. The three most intense peaks were subjected to higher energy collisional dissociation MS/MS. (c) The high-resolution tandem mass spectrum of the quadruply deprotonated anion at  $m/z$  1769.219 (the peak indicated by an arrow in panel b). The complete sequence was determined manually using the 5'-nucleotide-containing a, a-B, and c ions and 3'-nucleotide-containing w and y ions. Assignments are shown in the figure, and the sequence was identified as that of miR-21 by a BLAST search against the nr database. Source: Nakayama et al. (2015). Reproduced with permission of American Chemical Society.

difference between DNA and RNA fragmentation for sequencing using MS/MS. In the DNA the dominant fragment ions are a-B and w ions (McLuckey and Habibi-Goudarzi, 1993; McLuckey et al., 1992), and in the RNA the dominant fragment ions are c and y ions (Kirpekar and Krogh, 2001).

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