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Capillary Electrophoresis–Mass Spectrometry in Metabolomics: The Potential for Driving Drug Discovery and Development

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Abstract: Metabolomics represents the global profiling of metabolites in a given biological system. It has been used to facilitate pharmaceutical discovery and development for over a decade. Advances in instrumentation have positioned capillary electrophoresis–mass spectrometry (CE-MS) as an important platform for both targeted and non-targeted metabolomics. In this mini-review covering the last five years, we focus on the latest development of CE-MS based metabolomics in (a) identification and validation of novel valuable therapeutic targets, (b) evaluation of drug efficacy, and (c) monitoring of drug unanticipated adverse effects. Some of the current issues and future directions of CE-MS metabolomics are also discussed in the end.

Keywords: Biomarkers, CE-MS, drug discovery and development, drug efficacy, drug toxicity metabolomics.

1. INTRODUCTION

Drug discovery and development is a highly risky, time-consuming and expensive process that includes target identification, lead compound screening and optimization, preclinical development, clinical development (Phase I-III) and post-approval studies (Fig. **1**). At any of the stages, drug development might be terminated. The cost of drug development failure grows exponentially over the years in pharmaceutical companies [1]. Metabolomics potentially offers a solution to facilitate the drug development process and reduce the risks at all stages of the drug development pipeline. This technique aims to analyze all the small molecule metabolites (≤ 1500Da) in a given biological system. Metabolomics has been performed on a variety of analytical platforms such as NMR, GC-MS, LC/MS and CE-MS. The rapid analysis, efficient separation and low organic solvent consumption of CE in combination with the high sensitivity of the MS detector have positioned CE-MS as a powerful tool for pharmaceutical discovery. CE-MS is particularly suitable for the separation of complex mixtures of cationic, anionic, and/or zwitterionic metabolites, as well as their isobaric/isomeric ions without complicated sample handling in comparison to other analytical techniques. Over the past five years (2008-2013), the applicability of CE-MS based metabolomics has gradually gained acceptance in both academics and pharmaceutical companies to advance and facilitate drug discovery and development. Some of the well-known CE-MS based metabolomics groups from all over the world were listed in Table **1**. CE-MS based metabolomics has been used to support all of the drug development stages from screening drug targets, evaluating drug efficacy to tracking drug toxicity and adverse effects. Technical aspects of CE-MS metabolomics have recently been summarized by several groups [2-4]. In this mini-review, we discuss the latest and important advances of CE-MS based metabolomics in the support of drug discovery and development.

2. CE-MS METABOLOMICS IN DRUG TARGET IDENTIFICATION

One of the greatest advantages of metabolomics is that it can be performed in a non-targeted fashion. This is particularly useful in the screening of novel drug targets through metabolic profiling. The global metabolic profiling leads to the detection of metabolites with significant changes in a given disease and these metabolic biomarkers are then mapped to the specific biochemical pathways. The drug targets can be identified based on the enzymes or genes associated with the diseases in the identified pathways (Fig. **2**). For an easy overview, the applications of CE-MS based metabolomics on biomarkers discovery and drug target screening in the last five years (2008-2013) are listed in Table **2**. The table provides information about the sample type, types of diseases, metabolic markers and pathways. The details for several examples are discussed below.

CE-MS based metabolomics has identified glycolysis, tricarboxylic acid (TCA) cycle, pentose phosphate, purinergic signaling, amino acids metabolism pathway and urea cycle as important drug targetable biological pathways for various diseases Table **2**. For example, using CE-TOF-MS, Soga and coauthors performed a comprehensive profiling of 248 serum samples from 9 types of liver diseases and healthy controls [5]. They successfully identified γ-glutamyl dipeptides as potential biomarkers with remarkable increase in liver diseases. Interestingly, the concentration of γ-glutamyl dipeptides also has the power to distinguish among different types of liver diseases. Pathway analysis further showed that γ-glutamyl dipeptides are involved in the glutathione metabolism pathway. The discovery of such novel biomarkers may lay the ground work for the early diagnosis of liver disease, monitoring drug efficacy and designing new targets for liver disease treatment.

Another recent example of this approach is the assessment of metabolic changes in lung and prostate cancer. CE-TOF-MS metabolomics was carried out to quantify over 100 metabolites in the tissues of lung and prostate cancer patients [6]. Significantly high concentrations of the glycolytic pathway and TCA intermediates were observed in all tumor tissues. Enzyme analysis indicated that the hyperactive glycolysis and TCA are associated with the elevated activation of phosphofructokinase and pyruvate kinase phosphorylation. This finding might lead to the development of more effective cancer therapeutics.

Recently, a freely available database (Mouse Multiple Tissue Metabolome Database, MMMDB) that is specific for CE-MS metabolomics was developed by a group in Japan [7]. MMMDB (http://mmmdb.iab.keio.ac.jp.) contains more than 200 endogenous metabolites in multiple tissues of a single mouse that were quanti-

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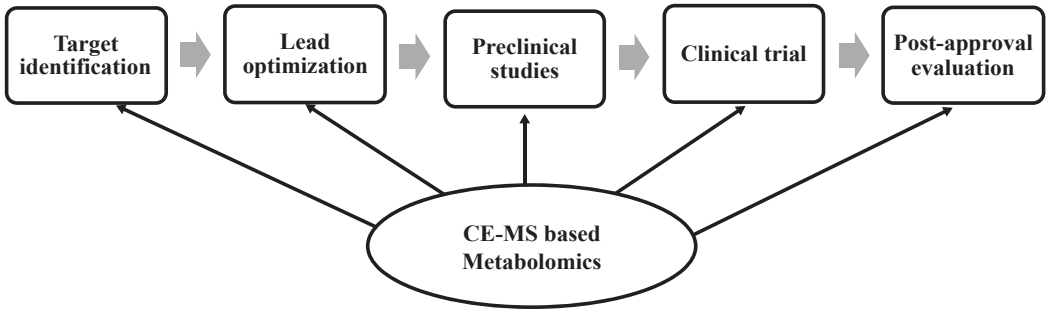

Fig. (1). Stages in the drug discovery and development (R&D) process.

Table 1. Some of the CE-MS metabolomics groups in academics and industry.

Groups	Country	Principal Investigator	Link
Human Metabolome Technologies Inc (HMT)	Japan	Masaru Tomita	http://humanmetabolome.com/
Institute of Advanced Biosciences, Keio University	Japan	Tomoyoshi Soga	http://metabolome.iab.keio.ac.jp/
Center for proteomics and metabolomics, LUMC	The Netherlands	André M. Deelder	https://www.lumc.nl/con/1040/81028091348221/811071049172556/
Center for Metabolomics and Bioanalysis, Universidad San Pablo CEU	Spain	Coral Barbas	http://www.metabolomica.uspceu.es/
Laboratory of Foodomics, CIAL (CSIC)	Spain	Alejandro Cifuentes	http://www.cial.uam-csic.es/pagperso/foodomics/
Department of Chemistry, McMaster University	Canada	Philip Britz-McKibbin	http://www.chemistry.mcmaster.ca/britz/pioverview.htm
Department of Chemistry and the Beckman Institute, University of Illinois at Urbana-Champaign (UIUC)	US	Jonathan V. Sweedler	http://www.scs.illinois.edu/sweedler/CE-MS.html

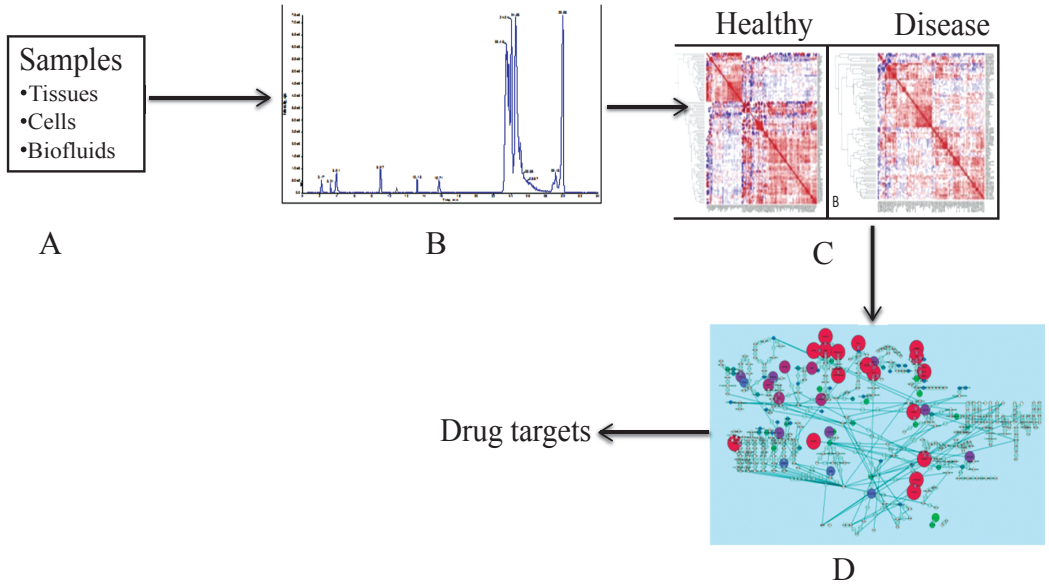

Fig. (2). The identification of drug targets through CE-MS based metabolomics. (**A**) Metabolites were extracted from biological samples (**B**) Metabolomics analysis using CE-MS (**C**) Multivariate analysis of metabolic differences between healthy and disease groups (**D**) Mapping of metabolites with significant changes into biological pathways.

fied by CE-TOF-MS. For each metabolite, the database has the molecular structure, formula, Kyoto Encyclopedia of Genes and Genomes (KEGG) ID, tissue source, concentration in a specific tissue, electropherograms, migration time in CE and mass spectrum.

3. CE-MS METABOLOMICS IN DRUG EFFICACY EVALUATION

Drug efficacy evaluation is one of the most critical areas for drug screening and development. The proof of drug efficacy might take months or even years. Metabolomics is well positioned to

Table 2. The applications of CE-MS metabolomics in diagnostic biomarkers and drug targets screening.

Pathology	Sample type	Metabolic markers	Potential Pathways	References
Liver diseases	Human serum	γ-glutamyl dipeptides	Production of reduced glutathione	[5]
Lung and prostate tumor	Human tumor tissues	TCA cycle intermediates such as malate, fumarate, succinate and essential amino acids inlcuding asparagine, lysine, phenylalanine, serine	Glycolytic and TCA cycle	[6]
Colon cancer	Cells from cancer patients	Reduced glutathione/oxidized glutathione (GSH/GSSG); polyamines	Urea cycle	[15]
Alzheimer's disease	Human cerebrospinal fluid	Choline, dimethylarginine, valine, proline	NA	[30]
Oral, breast and pancreatic cancer	Human saliva	Choline, arginine, creatinine phosphate, leucine, valine, tryptophan, polyamines and other 47 unknown compounds	Choline metabolism	[31]
Oxidative stress	Human plasma	Thiol redox (GSH/GSSG,Cysteine/cystine, and other	Thiol-redox pathways	[32]
Fast-Aging	Mice urine	Histidine, S-adenosyl-L-methionine, lysine, N-acetylspermidine, Trimethyl-L-Lysine	NA	[33]
Circadian rhythm disorder	Mice plasma	Carnitine, cytidine, hydroxyproline, creatinine, sarcosine, valine, glycine and other 21 compounds	Creatine pathway and glycine metabolism	[34]
Oxidative stress	Human red blood cells	GSH/GSSG	Glutathione metabolism	[35]
Regional pain syndrome	Human cerebrospinal fluid, urine and plasma	Citric acid, lactic acid and pyroglutamic acid and amino acids (lysine, arginine, valine, tyrosine and methionine)	NA	[36]
Colon and stomach cancer	Tumor tissues and cancer cells from patients	Lactate, all amino acids except glutamine	Glycolysis, Pentose phosphate, TCA and urea cycle	[37]
Colorectal Cancer	Human urine	Isoleucine, valine, arginine, lactate acid, histidine, methionine, serine, aspartic acid, citric acid, succinate, malic acid and leucine	Glycolysis, TCA, serine metabolism	[38]
Ventilator-induced lung injury	Rat serum	Asymmetric dimethyl arginine (ADMA), ornithine, arginine, choline, leucine/isoleucine	NA	[39]

speed up the process and can help the evaluation in three ways: the detection of drug mode of action, identification of diagnostic biomarkers and assessment of the recovery of whole body metabolism. This could save millions of dollars in development costs [8]. Indeed, in recent years, there has been an increasing amount of literature on the use of CE-MS metabolomics to aid drug efficacy evaluation.

It is well accepted that oxidative stress is involved in the progression of diabetes and its complications [9]. The increased production of free radicals is usually detected in patients with diabetes due to oxidation of glucose. Antioxidant therapy has been used as a complementary therapeutic approach for diabetes [10]. Cystoseira is a genus of brown algae containing high levels of antioxidants such as fucoxanthin and other carothenes. In order to evaluate the efficacy of *Cystoseira* spp extract on the treatment of type I diabetes, CE-MS based metabolomics was performed to characterize the metabolic differences in urine before and after the administration of *Cystoseira* extract to streptozotocin (STZ) diabetic rats [11]. The results showed that lysine metabolism was strongly altered by diabetes. Fructosyl-lysine markedly increased in STZ diabetic rats compared with normal healthy rats. However, the fructosyl-lysine level was reduced to normal range after administration of *Cystoseira* spp extract.

Rosmarinus officinalis is a woody medicinal herb used as spice and folk medicine around the world [12]. Recently, the efficacy of *R. officinalis* on the treatment of type I diabetes was evaluated by CE-MS based metabolomics using the STZ diabetic rats model [13]. Untargeted metabolomics profiling of urine samples with and without treatment was carried out using CE-TOF-MS for both control and diabetic rats. In total 6545 metabolic features were detected by CE-TOF-MS. Orthogonal projection to latent structures discriminant analysis (OPLS-DA) completely separated diabetic controls from diabetic rats treated with *R. officinalis* extract (Fig. **3**). This indicated that *R. officinalis* extract has significant impact on STZ diabetic rats' metabolism. The authors further identified 229 features with significant differences between control and treatment groups by matching *m/z* and MS/MS to the information in the Metabolite and Tandem MS Database (METLIN), KEGG and Human Metabolome Database (HMDB). Their metabolomics results showed a 37.8-fold reduction in the level of hexosamine in diabetic rats treated with *R. officinalis* extract. Hexosamine has been suggested to be one of the mechanisms that mediate glucose-induced insulin resistance [14]. In addition, reduction of 2-aminobutyric acid, leucine/isoleucine and dimethylglycine were also detected. Overall, the metabolomics results along with the reduction in diuresis and plasma triglycerides have demonstrated that *R. officinalis* extract is able to reduce some of the complications occurring in STZ diabetic rats.

Additionally, the effect of *R. officinalis* extract on human colon cancer cells proliferation was evaluated by CE-TOF-MS metabolomic analysis [15]. A total of 212 metabolites in colon cancer cells were found to be significantly different after *R. officinalis*

extract treatment. These metabolites with significant changes were mapped to biological pathways. The results showed that glutathione and polyamine metabolism had changed in response to *R. officinalis* extract treatment. An increase of glutathione/oxidized glutathione (GSH/GSSG) ratio was detected in *R. officinalis* extract treated colon cancer cells.

Another interesting example was the study of the mode of action of the antimonial drug Sb (III) potassium tartrate. The metabolic fingerprint of the parasite *Leishmania infantum* treated with 120 μM of Sb (III) potassium tartrate was obtained by CE with a sheathless interface and electrospray ionization (CE-ESI TOF-MS) and compared with control groups without treatment [16]. The authors found that sulfur containing amino acids and metabolites in the polyamine biosynthesis pathways were significantly decreased in *Leishmania infantum treated with* Sb (III) potassium tartrate. Their results also suggested that Sb (III) might disrupt the thiol-dependent redox metabolism in *Leishmania infantum*. This metabolomics study provided useful information for the anti-parasiticmechanism of Sb (III).

D-Glucosamine hydrochloride (GlcN·HCl) is widely used in the treatment of joint diseases in humans as well as animals. It promotes the regeneration of articular cartilage [17]. Osaki and co-authors investigated the mode of action of GlcN·HCl through CE-MS based metabolomics [18]. By quantifying the metabolites concentrations in dog plasma before and after oral administration of GlcN·HCl, they detected a sharp increase of succinic acid, malic acid, lactic acid and pyruvic acid after drug treatment. In addition, the levels of fumaric acid, hydroxyproline and alanine were significantly higher after oral administration of GlcN·HCl than before. Further pathway analysis indicated that GlcN·HCl might induce anaerobic respiration which leads to the accumulation of lactic acid and alanine. The rise of lactic acid induces the production of TGF-β, a cytokine with a function related to collagen production and thus stimulates cartilage regeneration (Fig. **4**).

The CE-MS metabolomics approach was also used to evaluate the absorption capacity of AST-120 towards uremic toxins produced in chronic kidney disease (CKD) [19]. The authors performed quantitative metabolomic analysis of 26 plasma samples

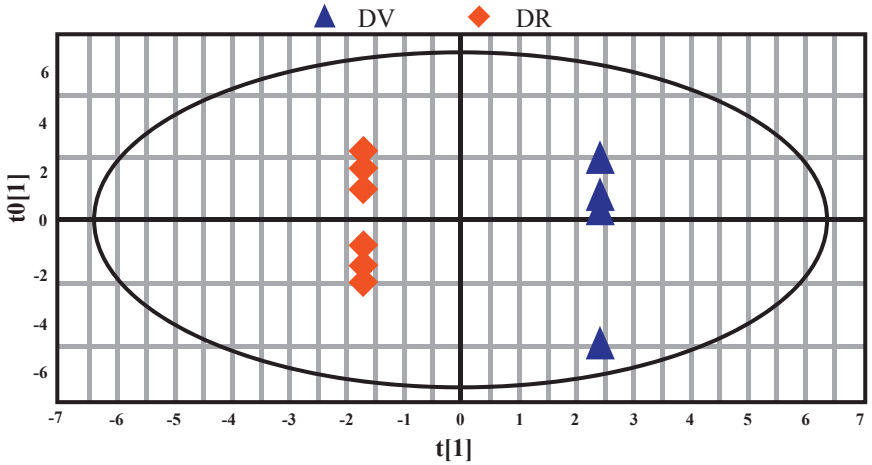

Fig. (3). The effect of *Rosmarinus officinalis* extract on urine metabolism of diabetic rats. Untargeted metabolomic analysis was performed by CE-TOF-MS. The data set was analyzed by OPLS-DA. DV: indicates diabetic rats without drug treatment (Control groups). DR: indicates diabetic rats treated with *Rosmarinus officinalis* extract. Figure was adapted from [13] and reproduced with permission.

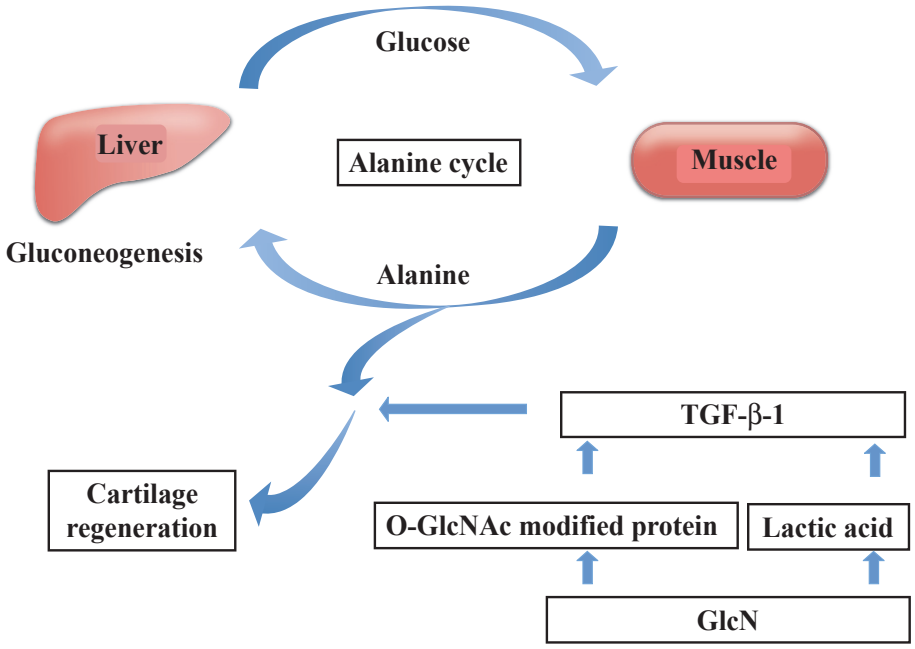

Fig. (4). Mode of action of D-Glucosamine hydrochloride (GlcN·HCl) evaluated by CE-MS based metabolomics. Figure was adapted from [18] and reproduced with permission.

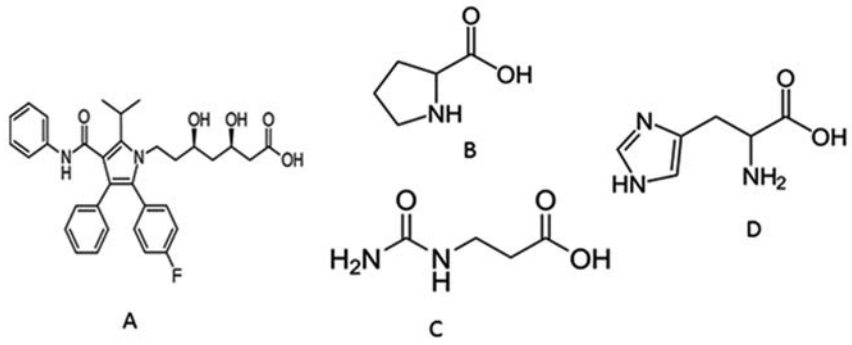

Fig. (5). The structure of atorvastatin and its toxicity biomarkers in urine. **A**: atorvastatin; **B**: proline; **C**: 3-ureidopropionic acid; **D**: histidine.

from CKD model rats. In total220 anionic and 300 cationic compounds were quantified and 23 compounds out of 520 were significantly decreased in response to AST-120 treatment. Among these 23 compounds, the plasma levels of *N*-acetyl-neuraminate, 4-pyridoxate, 4-oxopentanoate, glycine and 7 other molecules were significantly increased in the CKD model rats. These 10 compounds could represent good biomarkers for monitoring the AST-120 efficacy.

4. CE-MS METABOLOMICS IN DRUG TOXICITY MONITORING

The earliest inroads that metabolomics made into drug development and discovery is in the area of drug toxicity monitoring [20]. One of the advantages of metabolomics is that it provides a high throughput approach for rapid toxicity screening. This toxicity monitoring can be performed simultaneously with a dosage response study using the same sample and the same testing platform. Traditional compound safety screening only evaluates drug toxicity predicted by the chemical structures or other physical and chemical properties. In contrast, metabolomics provides a comprehensive assessment on the whole metabolism and might lead to discovery of unexpected side effects of a drug. Biomarkers or biomarker profiles that may be characteristic of physiological stress, tissue damage, tissue specific toxicity or general toxicity could be identified through metabolomics. Many of these toxicity biomarkers or biomarker profiles identified from animals models could also be detected and interpreted in humans. In this way, it may be possible to inexpensively detect an adverse response well in the early stage of drug development. Recent work demonstrated how CE-MS based metabolomics can be used for safety assessment of a drug.

Atorvastatin is a synthetic 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitor used for the treatment of hyperlipidemia and prevention of coronary artery disease. In an exploratory study of potential adverse effects of atorvastatin, urine targeted metabolomics was conducted using CE-MS [21]. The authors found that most of the amino acids in urine increased under hyperlipidemic conditions and decreased after drug treatment. In contrast, the urinary levels of proline, 3-ureidopropionic acid and histidine increased significantly in a dose-dependent manner in response to atorvastatin. This result suggested that these three amino acids could be used as metabolic markers for liver toxicity caused by atorvastatin. The structure of atorvastatin and its toxicity biomarkers in urine is shown in (Fig. **5**).

Selenomethionine (SeMet) is a naturally occurring amino acid containing selenium and is being used for cancer chemoprevention in several clinical trials [22]. The cytotoxicity of SeMet was evaluated on yeast using CE-MS based metabolomics by Kitajima and coauthors [23]. Global metabolic profiling using CE-TOF-MS led to the detection of 221 compounds in the yeast metabolome. Thirteen metabolites out of 221 were significantly changed following exposure to SeMet and most of them were thiol compounds. This suggested that the cytotoxicity of SeMet might be due to the disruption of thiol metabolism in the living cells.

Non-steroid anti-inflammatory drugs (NSAIDs) are the common prescriptions for the treatment of rheumatoid arthritis, osteoarthritis, acute pain and fever [24]. Recently, the metabolome differential display method based on CE−MS was successfully applied to identify the candidate biomarkers of NSAIDs-induced gastric injury [25]. The rapid analysis and efficient resolution with high sensitivity of CE-MS allows the authors to simultaneously detect 580 peaks in a single run (within 45 min). Significantly decreased levels of TCA cycle intermediates citrate, cis-aconitate and succinate were observed in response to the increase of NSAIDs concentration. In addition, NSAIDs also induced a decrease of 3-hydroxy butyric acid and o-acetyl carnitine which are involved in β-oxidation in mitochondria. These candidate biomarkers might have clinical importance in helping to monitor the side effects of aspirin, ibuprofen and naproxen which are common NSAIDs.

5. CONCLUDING REMARKS AND OUTLOOK

The use of CE-MS metabolomics for drug discovery and development has increased considerably over the last few years. It has truly revolutionized the way drugs are developed. As the examples highlighted in this mini-review, CE-MS metabolomics has implications not only for therapeutic target screening, drug efficacy evaluation but also for toxicity monitoring.

However, compared to other analytical platforms, the application of CE-MS in metabolomics is still limited. From a technical point of view, this might be due to the reproducibility of migration time and sensitivity of CE. The new development of novel interfaces for CE and MS coupling and the use of in-capillary preconcentration techniques enable the scientists to perform the global profiling of biological samples at a system level [26-28]. The low sample consumption typical for CE is a clear advantage for CE-MS based metabolomics and leads to the emergence of single-cell metabolomics which provides a new perspective for the entire metabolomics field [29].

Another technical difficulty that hampers the application of metabolomics in drug discovery is the annotation of unknowns. Currently, untargeted metabolic profiling generates many more unknowns than knowns. For example, typically, more than 2000 features can be obtained in a single run of metabolic profiling of a plasma sample using CE-TOFMS. However, only about 200 features (~10%) can be identified by matching the closest *m/z* in the databases. Public databases and their collections are still limited. Currently, only MMMDB is specific for CE-MS based metabolomics. Scientists in the CE-MS metabolomics field are encouraged to deposit their original data including experimental conditions, electropherograms, migration time and mass spectrum information into a publicly available repository.

CE-MS based metabolomics has successfully identified many metabolic biomarkers which significantly facilitated drug R&D processes. However, some of the identified biomarkers are too general and belong to the common cell defense metabolism that can be found in many disorder. More specific, informative and reliable biomarkers are needed. It is also necessary to cross-validate the biomarkers identified by CE-MS using different metabolomics platforms or other "omics" techniques such as proteomics and transcriptomics.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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