ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/5385611

Towards Absolute Quantification of Therapeutic Monoclonal Antibody in Serum by LC–MS/MS Using Isotope-Labeled Antibody Standard and Protein Cleavage Isotope Dilution Mass Spectrome...

ARTICLE *in* ANALYTICAL CHEMISTRY · JULY 2008

Impact Factor: 5.64 · DOI: 10.1021/ac800205s · Source: PubMed

CITATIONS
113

READS
223

8 AUTHORS, INCLUDING:

Samuel Barteau
Novartis
8 PUBLICATIONS **222** CITATIONS
SEE PROFILE

Dieter Zimmer
Zimmer BioAnalytics & More
23 PUBLICATIONS **471** CITATIONS
SEE PROFILE

Olivier Kretz
Novartis Institutes for BioMedical Research
39 PUBLICATIONS **607** CITATIONS
SEE PROFILE

Available from: Samuel Barteau
Retrieved on: 29 January 2016

Anal. Chem. **2008,** *80,* 4200–4207

Towards Absolute Quantification of Therapeutic Monoclonal Antibody in Serum by LC−MS/MS Using Isotope-Labeled Antibody Standard and Protein Cleavage Isotope Dilution Mass Spectrometry

Olivier Heudi,*,† Samuel Barteau,† Dieter Zimmer,† Joerg Schmidt,‡ Kurt Bill,‡ Natalie Lehmann,‡ Christian Bauer,‡ and Olivier Kretz†

DMPK/Bioanalytics, and Biotechnology Development, Novartis Pharma AG, CH-4002 Basel Switzerland

Although LC−MS methods are increasingly used for the absolute quantification of proteins, the lack of appropriate internal standard (IS) hinders the development of rapid and standardized analytical methods for both in vitro and in vivo studies. Here, we have developed a novel method for the absolute quantification of a therapeutic protein, which is monoclonal antibody (mAb). The method combines liquid chromatography tandem mass spectrometry (LC−MS/MS) and protein cleavage isotope dilution mass spectrometry with the isotope-labeled mAb as IS. The latter was identical to the analyzed mAb with the exception that each threonine contains four ^{13}C atoms and one ^{15}N atom. Serum samples were spiked with IS prior to the overnight trypsin digestion and subsequent sample cleanup. Sample extracts were analyzed on a C_{18} ACE column (150 mm × 4.6 mm) using an LC gradient time of 11 min. Endogenous mAb concentrations were determined by calculating the peak height ratio of its signature peptide to the corresponding isotope-labeled peptide. The linear dynamic range was established between 5.00 and 1000 μg/mL mAb with accuracy and precision within ±15% at all concentrations and below ±20% at the LLOQ (lower limit of quantification). The overall method recovery in terms of mAb was 14%. The losses due to sample preparation (digestion and purification) were 72% from which about 32% was due to the first step of the method, the sample digestion. This huge loss during sample preparation strongly emphasizes the necessity to employ an IS right from the beginning. Our method was successfully applied to the mAb quantification in marmoset serum study samples, and the precision obtained on duplicate samples was, in most cases, below 20%. The comparison with enzyme-linked immunosorbent assay (ELISA) showed higher exposure in terms of AUC and C_{max} with the LC−MS/MS method. Possible reasons for this discrepancy are discussed in this study. The results of this study indicate that our LC−MS/MS method is a simple, rapid, and precise approach for the therapeutic mAb quantification to support preclinical and clinical studies.

Therapeutic monoclonal antibodies (mAbs) represent one of the fastest-growing markets in biotechnology. At the end of 2006, 18 therapeutic mAbs have been approved by the U.S. Food and Drug Administration for the treatment of various diseases. Out of these 18 mAbs, 12 are IgG1 and 3 are IgG2.[1] The ability to quantify mAbs in biological fluids is important for (1) compound selection at the early stage of development and (2) evaluating the pharmacokinetic (PK) parameters, drug exposure toxicity, and pharmacodynamic relationships in order to support their preclinical and clinical studies. Hence, a reliable bioanalytical method with high selectivity, accuracy, precision, and short development period is highly desirable.

Immunoassays are extensively used for quantifying proteins in biological fluids to support preclinical and clinical studies.[2,3] Although these assays offer high sensitivity and throughput, the development of a specific antibody or antigen to the protein of interest is a time-consuming process. Furthermore, other proteins found in the analyzed biological fluid can interfere with the assay and give spurious PK profiles.

Liquid chromatography tandem mass spectrometry (LC−MS/MS) is currently one of the most promising analytical techniques, which combines a robust separation technique with identification and quantification. Furthermore, LC−MS/MS is accurate, precise, and enables throughput analysis. Several LC−MS/MS works have been dedicated to the absolute quantification of the intact protein in biological fluids[4–6] or in food.[7] However, the application of this approach is not straightforward for the analysis of larger proteins such as mAbs due to some limitations in instrumentation and in

* Corresponding author. E-mail: Olivier.heudi@novartis.com. Phone: +41 61 696 41 07. Fax: +41 61 696 58 65.

† DMPK/Bioanalytics.

‡ Biotechnology Development.

(1) Schrama, D.; Reisfeld, R. A.; Becker, J. C. *Nat. Rev. Drug Discovery.* **2006**, *5*, 147–159.

(2) DeSilva, B.; Smith, W.; Weiner, R.; Kelley, M.; Smolec, J. M.; Lee, B.; Khan, M.; Tacey, R.; Hill, H.; Celniker, A. *Pharm. Res.* **2003**, *20*, 1885–1900.

(3) Dubois, M.; Delaunay, V.; Delestre, L.; Ezan, E. *J. Pharm. Biomed.* **2007**, *43*, 1423–1429.

(4) Becher, F.; Pruvost, A.; Clement, G.; Tabet, J. C.; Ezan, E. *Anal. Chem.* **2006**, *78*, 2306–2313.

(5) Ji, Q. C.; Gage, E. M.; Rodila, R.; Chang, M. S.; El-Shourbagy, T. A. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 794–799.

(6) Ji, Q. C.; Rodila, R.; Gage, E. M.; El-Shourbagy, T. A. *Anal. Chem.* **2003**, *75*, 7008–7014.

(7) Czerwenka, C.; Maier, I.; Potocnik, N.; Pittner, F.; Lindner, W. *Anal. Chem.* **2007**, *79*, 5165–5172.

10.1021/ac800205s CCC: $40.75 © 2008 American Chemical Society
Published on Web 05/09/2008

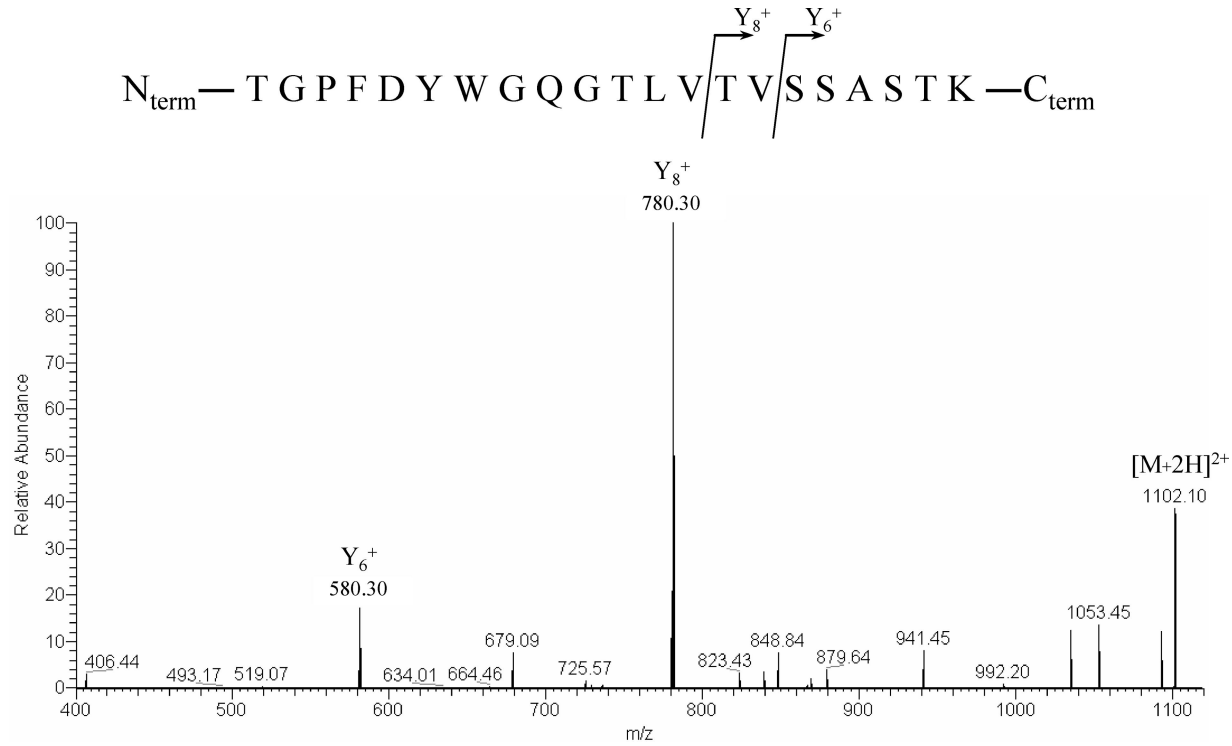

Figure 1. Peptide signature sequence and its MS fragmentation pattern.

Table 1. Molecular Weight of the Nonlabeled mAb (Analyte) and Labeled mAb (IS) with ^{13}C and ^{15}N Isotopes

	light chain [Da]	heavy chain [Da]
nonlabeled mAb	23 643.26	49 737.08
no. of threonines	17	33
mass difference per isotope-labeled threonine	4.96	4.96
mass increase of mAb by labeled threonine	84.32	163.68
isotope-labeled mAb	23 727.58	49 900.76

the ionization process. Therefore, the quantification of larger proteins would require additional steps in the sample preparation. Immunoprecipitation capture of the proteins of interest followed by the trypsin digestion prior to LC−MS/MS analysis has been investigated.[8–10] One drawback of this approach is the time required to produce a specific antibody or antigen to the protein of interest. The use of proteolysis coupled with MS for the quantification of apolipoprotein A-1 was proposed by Barr et al.[11] In their work, the protein of interest was digested with trypsin and quantified using a stable isotope labeled internal standard peptide with LC-flow−FAB/MS/MS. Other groups have applied this approach in human serum for the quantification of prostate-specific antigen,[12] intermediate abundance proteins,[13] C-reactive protein,[14] and apolipoproteins.[15] With this approach the internal standard (IS), an isotope-labeled peptide, is added to the samples after the completion of the trypsin digestion step. In practice, this is not adequate for full-length protein losses or incomplete enzyme digestion that may occur during the sample processing. To circumvent this problem, analogue proteins have been used for the quantification of myoglobin in human serum[16] or mAb in rat serum.[17] Also, labeled artificial concatemers of standard peptides have been recently used as IS for the protein quantification.[18–20] However, analogue proteins or concatemer peptides are not the ideal match for the biochemical properties of the analyzed protein. Therefore, it is not possible to correct for losses during the sample preparation and especially any recovery differences throughout the entire analytical process. Previous works on the absolute quantification of rheumatoid arthritis biomarker have demonstrated that the recovery of the protein from serum was only 60%.[14] In another study, dealing with the absolute quantification of four serum proteins by LC−MS/MS, it has been shown that significant protein losses can occur during the sample preparation.[13] To

(8) Becher, F.; Duriez, E.; Volland, H.; Tabet, J. C.; Ezan, E. *Anal. Chem.* **2007**, *79*, 659–665.
(9) Dubois, M.; Becher, F.; Herbet, A.; Ezan, E. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 352–358.
(10) Hoos, J. S.; Sudergat, H.; Hoelck, J. P.; Stahl, M.; de Vlieger, J. S. B.; Niessen, W. M. A.; Lingeman, H.; Irth, H. *J. Chromatogr., B* **2006**, *830*, 262–269.
(11) Barr, J. R.; Maggio, V. L.; Patterson, D. G.; Cooper, G. R.; Henderson, L. O.; Turner, W. E.; Smith, S. J.; Hannon, W. H.; Needham, L. L.; Sampson, E. J. *Clin. Chem.* **1996**, *42*, 1676–1682.
(12) Barnidge, D. R.; Goodmanson, M. K.; Klee, G. G.; Muddiman, D. C. *J. Proteome Res.* **2004**, *3*, 644–652.
(13) Lin, S. H.; Shaler, T. A.; Becker, C. H. *Anal. Chem.* **2006**, *78*, 5762–5767.
(14) Kuhn, E.; Wu, J.; Karl, J.; Liao, H.; Zolg, W.; Guild, B. *Proteomics* **2004**, *4*, 1175–1186.
(15) Gay, R.; Gregory, B.; Grace, P.; Pleasance, S. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2585–2593.
(16) Mayr, B. M.; Kohlbacher, O.; Reinert, K.; Sturm, M.; Gropl, C.; Lange, E.; Klein, C.; Huber, C. G. *J. Proteome Res.* **2006**, *5*, 414–421.
(17) Yang, Z.; Fang, X.; Hayes, M.; Daley, M. P.; Ettenberg, S.; Tse, F. L. S. *Anal. Chem.* **2007**, *79*, 9294–9301.
(18) Anderson, L.; Hunter, C. L. *Mol. Cell. Proteomics* **2006**, *5*, 573–588.
(19) Beynon, R. J.; Doherty, M. K.; Pratt, J. M.; Gaskell, S. J. *Nat. Methods* **2005**, *2*, 587–589.
(20) Rivers, J.; Simpson, D. M.; Robertson, D. H. L.; Gaskell, S. J.; Beynon, R. J. *Mol. Cell. Proteomics* **2007**, *6*, 1416–1427.

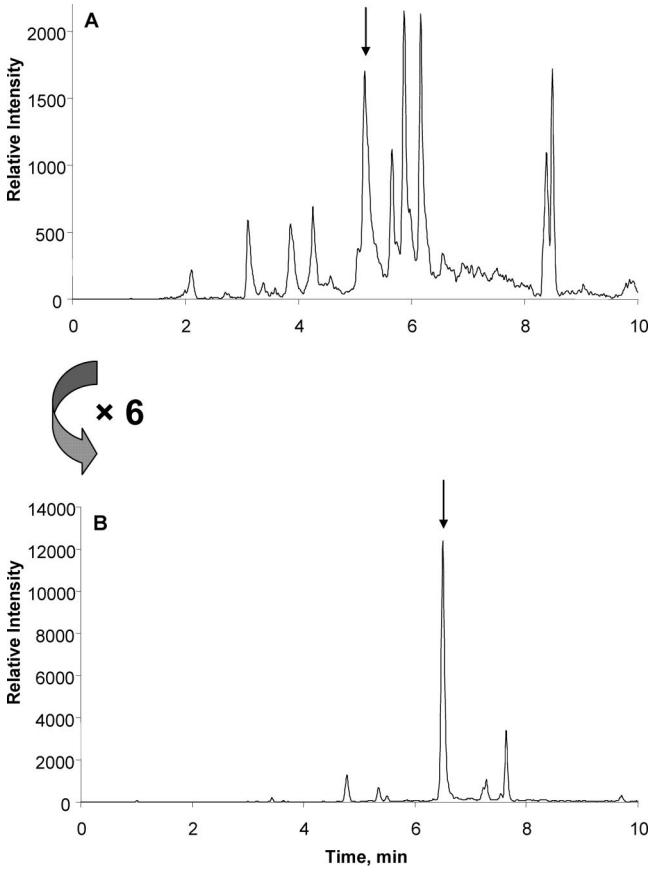

Figure 2. Comparison of signature peptide MRM channels of an extracted serum sample spiked with mAb at 50 μg/mL obtained with (A) an Agilent Zorbax Eclipse XD C_8 (50 mm × 2.1 mm, 3.5 μm particle) and (B) an ACE C_{18} (150 mm × 4.6 mm, 3.0 μm particle). The arrow indicates the signature peptide and IS retention time.

enable a reliable MS quantification method, the use of isotope-labeled IS is highly desirable. Recently, Brun et al.[21] have developed the protein standard absolute quantification (PSAQ) as an innovative approach. The latter used in vitro synthesized isotope-labeled full-length proteins as IS and MS for the absolute quantification of biomarkers. Their results demonstrated the advantage of the PSAQ approach over conventional methods which either used labeled tryptic peptides or concatemer as IS. In a recent published study, it has been shown that isotope-labeled protein standards are predicted to be particularly useful for the measurement by LC−MS/MS of highly homologous isoenzymes such as alcohol dehydrogenase using a protein cleavage method.[22] The additional benefits of isotope-labeled protein as IS in comparison to a chemically synthesized signature peptide is that, during method development, the signature peptide (the peptide used for the LC−MS/MS quantification) can be chosen and changed. Moreover, several signature peptides of the analyzed proteins can be monitored within the same run. To our knowledge, no study describing the use of isotope-labeled protein as IS for the LC−MS/MS absolute quantification of mAbs has been found in the literature.

(21) Brun, V.; Dupuis, A.; Adrait, A.; Marcellin, M.; Thomas, D.; Court, M.; Vandenesch, F.; Garin, J. *Mol. Cell. Proteomics* **2007**, *12*, 2139–2149.
(22) Janecki, D. J.; Bemis, K. G.; Tegeler, T. J.; Sanghani, P. C.; Zhai, L.; Hurley, T. D.; Bosron, W. F.; Wang, M. *Anal. Biochem.* **2007**, *369*, 18–26.

In this publication, we report experimental data of a novel protein cleavage isotope dilution method for the quantification of mAbs in marmoset serum by LC−MS/MS assay with the isotope-labeled mAb as IS. As a proof of concept, we have used one of our mAb drug candidates, which is a novel mAb for the treatment of rheumatoid arthritis. In this work, the IS used for the quantification was identical to the analyzed mAb but each threonine contained four ^{13}C atoms and one ^{15}N atom.

EXPERIMENTAL SECTION

Materials. The production of nonlabeled and labeled mAbs followed the same procedure except that for the labeled mAb (IS) the threonine added into the medium was labeled with ^{13}C and ^{15}N atoms. Typically, SP2/0 Ag 14.0 cells were cultivated in CHO basal medium (Irvine Scientific, Santa Ana, CA) under standard conditions. The mAbs were then purified by protein A chromatography followed by anion-exchange chromatography on Q-Sepharose fast flow.

The synthetic signature peptide was purchased from NeoMPS (Strasbourg, France). HPLC grade methanol, ethanol (absolute), acetic acid, trifluoroacetic acid (TFA), and tetrahydrofuran (THF) were obtained from Merck KGaA (Darmstadt, Germany). Formic acid, mercaptoethanol, and Tris buffer (pH 8.1) were obtained from Fluka (Buchs, Switzerland). Guanidinium chloride was from Pierce (Rockford, IL). Ammonium bicarbonate, trypsin (TPCK treated) from bovine pancreas, urea, ammonium hydroxide, hydrochloric acid (1 M in water), DL-dithiothreitol (DTT), and iodoacetamide were obtained from Sigma-Aldrich (St. Louis, MO). Carboxypeptidase B and PNGase F were from Roche Diagnostics (Mannheim, Germany) and New England Biolabs (Ipswich, MA), respectively. MilliQ grade water was produced by a Millipore system (Bedford, MA). All other HPLC solvents were of LC−MS grade from Baker (Deventer, The Netherlands). The 96-well Oasis MCX LP plates 60 μm (30 mg) were obtained from Waters Inc. (Milford, MA). Marmoset serum batches were obtained from center de primatologie ULP (Strasbourg, France).

Instrumentation. The LC−MS/MS system used for the intact nonlabeled and labeled mAb molecular weight measurements consisted of a UPLC with an external column oven connected to a Q-ToF Ultima API (Waters, Milford, MA). The system was controlled by MassLynx 4.1 (Waters, Milford, MA).

The LC−MS/MS system used for the quantification work consisted of a Symbiosis Pharma 1.0.0.0 unit from Spark (Emmen, The Netherlands) equipped with a Reliance unit (conditioned stacker and autosampler). A TSQ Quantum ultra triple-quadrupole mass spectrometer from ThermoFischer (San Jose, CA) equipped with H-ESI and operating in positive-ion multiple reaction monitoring was used. Additionally, a 1200 series column compartment from Agilent Technologies (Wilmington, DE) was used as the column oven. Data acquisition was performed with Xcalibur software, version 2.0.

Preparation of Stocks, Working Standard, IS, and Quality Control (QC) Solutions. Individual mAb and IS stocks were prepared in 400 mM ammonium bicarbonate to give a final concentration of 2500 μg/mL. Individual working calibration standard solutions with concentrations of 11.5, 46.1, 115.3, 230.7, 692.3, and 2307.7 μg/mL were prepared after serial dilutions of the stock solution with 400 mM ammonium bicarbonate. The working QC solutions with concentrations of 15.0, 45.0, 1500, and

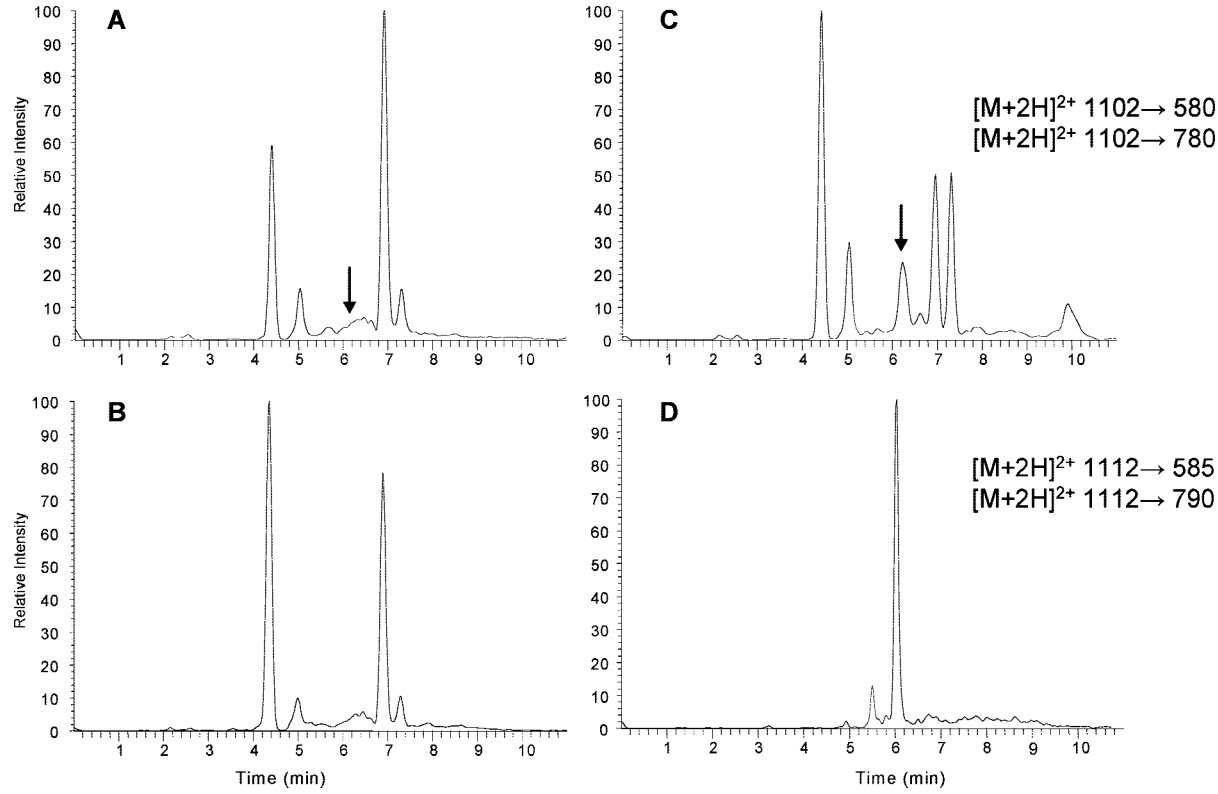

Figure 3. Reconstructed chromatograms showing the sum of the two MRM transitions of mAb for extracted double-blank serum sample (A), extracted blank serum sample spiked with IS (B), and extracted sample spiked with mAb at a concentration of 5 μg/mL (limit of quantification, LOQ) and IS (C). Reconstructed chromatogram showing the sum of the two MRM transitions of IS for extracted serum sample spiked with IS (D). The arrows indicate the signature peptide and IS retention time.

2250 μg/mL were also prepared in the same manner. The stocks and the working solutions were freshly prepared on each day of the analysis.

Preparation of Matrix Calibration and QC Samples. Two different serum batches were used for preparation of matrix calibration and QC samples. The calibration samples were prepared by spiking 65 μL of each mAb individual working calibration standard solution into 85 μL of blank serum. This yielded calibration standard concentrations of 5.00 (lower limit of quantification, LLOQ), 20.0, 50.0, 100, 300, and 1000 μg/mL. The QC samples were prepared by spiking 100 μL of each mAb working QC solution into 200 μL of blank serum. This gave final QC concentrations of 5.00, 15.0, 500, and 750 μg/mL.

Bioinformatics Tool for Identification of Unique Peptides in the mAb Sequence. To search for unique residues within the antibody heavy and light chains, a bioinformatics tool identified all related antibody sequences from human GenBank proteins. Antibody sequences were aligned, and the percent identity for each antibody residue position against the public antibody sequences was calculated. The tool provides an in silico tryptic digest, and for the identification of candidate signature peptides residues with low identity scores were singled out.

Sample Preparation. For the intact nonlabeled and labeled mAb molecular weight measurements, N-glycans were removed by PNGase F, followed by the sample's reduction and alkylation. Approximately 100 μg of mAb was incubated with PNGase F (10 μL of PNGase F per mg of mAb) in 100 mM Tris, pH 7.5 for 30 min at room temperature. Then NaCl (to give 100 mM) and a second portion of PNGase F (as above) were added, and the mixture was incubated for 4 h at 37 °C. The resulting mixture was diluted with GnCl/Tris buffer (6 M guanidinum chloride, 0.4 M Tris/HCl, pH 8). The mAb was reduced by the addition of 20 μL DTT (100 mM DTT in GnCl/Tris buffer) for 30 min at 50 °C under argon and then alkylated by adding 20 μL of iodoacetamide (240 mM iodoacetamide in GnCl/Tris buffer) for 15 min at room temperature in the dark. The excess reagent was inactivated by adding mercaptoethanol.

For the mAb quantification, serum samples were prepared as follows: to 50 μL of serum sample, 5 μL of IS at a concentration of 2500 μg/mL and 15 μL of 1 M DTT prepared in 20 M urea were added in a 96-well plate. The plate was incubated at 50 °C for 30 min in an HIS25 hybridization incubator from Grant Boekel (Cambridge, U.K.). A volume of 25 μL of 1 M iodoacetamide prepared in water was added, and the plate was incubated at room temperature in the dark for 90 min. Bovine trypsin was prepared at 3.5 mg/mL in 1 mM hydrochloric acid, and 0.7 μg trypsin/μL serum was added to the sample together with 700 μL of 1 M Tris buffer and 90 μL of HPLC grade water. The plate was capped, briefly centrifuged, and vortexed. Samples were digested overnight, and TFA 10% (v/v) was added the following day to terminate the digestion.

Digests were cleaned up by solid-phase extraction (SPE) using Oasis MCX 96-well plates. The plates were prewashed with 1.5 mL of methanol followed by 1.5 mL of HPLC grade water. A volume of 800 μL of digested sample was loaded and then washed with 2 × 1.3 mL of 1% acetic acid followed by 2 × 1.0 mL of methanol. Subsequently, 2 × 750 μL of 5% ammonium hydroxide in 50% methanol and 45% ethanol was used to elute into 96-well 2

Table 2. Intra- and Interday Precision (% CV) and Accuracy (% Bias) of the LC−MS/MS Method

concn (μg/mL)	5.00	15.0	500	750
n	5	5	4	5
intraday: day 1 % CV	16.4	9.17	8.29	2.35
intraday: day 1 % bias	5.56	0.05	−8.48	−4.81
n	5	5	5	5
intraday: day 2 % CV	17.2	12.8	6.54	7.51
intraday: day 2 % bias	8.41	5.16	−8.21	−10.3
n	10	10	9	10
interday % CV	15.9	10.9	6.87	6.01
interday % bias	6.99	2.61	−8.33	−7.57

mL collection plates. Samples were evaporated to dryness under a nitrogen stream at 55 °C. Prior to LC−MS/MS analysis, the dry extract was reconstituted with 100 μL of methanol/water (50:50 v/v) solution containing 0.1% formic acid.

Liquid Chromatography and Mass Spectrometry Conditions. For the intact nonlabeled and labeled mAb molecular weight measurements, chromatographic separation was performed at a flow rate of 0.12 mL/min on a POROS R2 column (Applied Biosystems, Foster City, CA) at 80 °C and separated with a shallow gradient of mobile phase A (0.05% TFA in water) and B (0.05% TFA in 90/10 acetonitrile/water). An amount of 10 μg of mAb was injected onto the column, and the mass spectrometer was operated in positive-ion mode. Collision energy, cone, and RF1 voltages were raised to a level just below where protein fragmentation occurred (15 eV, 60 V, and 80 V, respectively). The data, (1) mass calibration between each run and (2) light and heavy chain peaks deconvolution, were processed using MaxEnt 1 (10 iterations, MassLynx 4.1, Waters, Milford, MA). The expected masses were calculated by GPMAW 7.1 (Lighthouse Data, Odense, Denmark).

For LC−MS/MS quantification, chromatographic separation was performed at a flow rate of 1.0 mL/min on an ACE C_{18} column 150 mm × 4.6 mm, packed with 3 μm particles (Hichrom, Bershire, U.K.). A binary gradient was used to perform the separations. Mobile phase A consisted of water containing 0.1% formic acid, and mobile phase B consisted of methanol containing 0.1% formic acid. A 75 μL injection of each sample (injection loop volume, 100 μL) was loaded onto the column and eluted using the following gradient conditions [time (min), (% mobile phase B): (0, 30) (1, 30) (8, 95) (9.5, 95) (10, 30) (11, 30)]. The column temperature was maintained at 60 °C using a column heater. The autosampler syringe and the injection valve were washed with 1.5 mL of methanol/water (50/50) containing 0.1% formic acid, 1.5 mL of THF, and 1.5 mL of methanol/water (50/50) containing 0.1% formic acid, post sample injection, to reduce carryover. The system was operated in a positive-ion multiple reaction monitoring (MRM) mode. The signature peptide elicited pseudomolecular ions at multiple charge states in the ESI source, and the most abundant charge state was selected and fragmented in the collision cell. The MRM transition was established based on the formation of the two most abundant product ions. The MS conditions were as follows: collision energy 55 eV, sheath gas pressure 60 psi, auxiliary gas flow 60 arbitrary units, tube lens offset voltage 170 V (mode flat), spray voltage 4500 V, H-ESI probe vaporizer temperature 450 °C, and ion transfer tube temperature 350 °C.

Method Validation. Serum calibration curves were constructed using peak height ratios of mAb signature tryptic peptide to that of its IS and applying a weighted ($1/x$) linear regression analysis. Precision and accuracy were calculated for the QC samples at four concentration levels (5, 15.0, 500, and 750 μg/mL). Five replicates of each QC level were analyzed on each day of two days to determine the intraday accuracy and precision. Specificity was assessed by performing the analysis on a blank matrix sample (double blank) and on extracted blank matrix samples (spiked with the IS). The overall recovery of the method was calculated, at two concentrations (5.00 and 500 μg/mL mAb), as the mean peak height for the signature peptide in extract divided by the mean peak height for signature peptide in solution at the expected concentration ($n = 3$). The back calculation of mAb concentration was then performed. For the matrix effects assessment, as it was technically not possible to have a serum digest without the peptide signature, synthetic labeled signature peptide was used instead. The matrix effect was estimated, at two concentrations (5.00 and 500 μg/mL of mAb), by dividing the mean peak height for labeled synthetic peptide spiked in serum after digestion and extraction by an equal concentration of labeled synthetic peptide in neat solution ($n = 2$).

Table 3. Concentration (μg/mL) of mAb in Marmoset Serum Obtained by LC−MS/MS after Subcutaneous Administration of 50 mg/kg

	animal ID			
time [h]	3503a	3503b	mean	CV[a]
0	0	0	0	0
0.083	BLOQ[b]	6.52	6.52	N/A[c]
24	263	222	243	11.9
48	211	193	202	6.39
72	220	208	214	4.19
96	166	176	171	4.30
120	175	178	177	1.29
192	102	89.5	95.6	9.08
264	49.8	59.6	54.7	12.8
336	47.0	N/A	47.0	N/A
504	BLOQ	BLOQ	BLOQ	N/A
672	BLOQ	BLOQ	BLOQ	N/A
936	BLOQ	BLOQ	BLOQ	N/A
1008	BLOQ	BLOQ	BLOQ	N/A

[a] $n = 2$. [b] BLOQ: below the LLOQ (5.00 μg/mL). [c] N/A: not available.

Pharmacokinetics of mAb in Marmoset: in Vivo Experiment. Marmosets (three animals) were administered a single dose of 150 mg/kg mAb via subcutaneous injection. Whole blood samples of 500 μL were taken via the jugular vein cannula at 0, 0.083, 24, 48, 72, 96, 120, 192, 264, 336, 504, 672, 936, and 1008 h post dose. The blood was allowed to clot, then centrifuged at 3500 rpm, and the supernatant serum was separated. The samples were stored at −80 °C until analysis.

Sample Analysis Using Enzyme-Linked Immunosorbent Assay (ELISA). The serum samples were initially analyzed with an in-house validated method for mAb quantification, which is based on a competitive assay. Briefly, purified anti-idiotype anti-mAb was coated on a 96-well plate. Serum samples and biotin-labeled mAb were simultaneously incubated to compete for binding with the anti-idiotype anti-mAb. Bound biotinylated mAb was detected by incubating horseradish peroxidase-conjugated streptavidin with *O*-phenylenediamine dihydrochloride (OPD) as substrate.

Table 4. Concentration (μg/mL) of mAb in Marmoset Serum Obtained by LC−MS/MS after Subcutaneous Administration of 150 mg/kg

	animal ID				animal ID				animal ID			
time [h]	4502a	4502b	mean	% CV[a]	4503a	4503b	mean	% CV[a]	4504a	4504b	mean	% CV[a]
0	0.00	0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00	
0.083	N/A[b]	BLOQ[c]	BLOQ	N/A	21.7	17.6	19.7	14.6	BLOQ	BLOQ	BLOQ	N/A
24	959	N/A	959	N/A	1125	1136	1131	0.68	635	621	628	1.62
48	775	N/A	775	N/A	1109	1128	1119	1.15	562	N/A	562	N/A
72	N/A	N/A	N/A	N/A	965	982	973	1.22	N/A	N/A	N/A	N/A
96	560	596	578	4.34	843	856	850	1.08	480	453	467	3.99
120	558	536	547	2.82	739	629	684	11.4	376	398	387	3.99
192	344	468	406	21.5	337	395	366	11.2	228	194	211	11.3
264	212	229	221	5.62	299	348	324	10.7	161	170	166	3.66
336	162	182	172	8.29	186	148	167	16.3	98.3	94.6	96.4	2.73
504	70.6	64.3	67.5	6.62	94.4	84.7	89.5	7.63	33.3	31.3	32.3	4.41
672	22.2	28.3	25.2	17.0	35.6	38.1	36.8	4.94	13.9	14.7	14.3	3.73
936	N/A	N/A	N/A	N/A	14.7	13.5	14.1	5.72	BLOQ	BLOQ	BLOQ	N/A
1008	BLOQ	6.46	6.46	N/A	N/A	N/A	N/A	N/A	BLOQ	N/A	BLOQ	N/A

[a] $n = 2$. [b] N/A: not available. [c] BLOQ: below the LLOQ (5.00 μg/mL).

RESULTS AND DISCUSSION

Signature Peptide Selection and Identification. In silico analysis of the mAb sequence allowed us to identify unique tryptic signature peptides that could be potentially used for the quantification of the mAb. Multiple sequence alignment analysis determined identity counts for all amino acid residues with related GenBank antibody sequences. Residues that have a low identity score were regarded as unique. If the in silico search returned multiple peptides, some additional selection criteria were considered, for example, exclusion of peptides containing KK or RR sequences or having residues that are prone to chemical modification, such as Met, Trp, or Cys. Unstable sequences, such as Asp-Gly, and N-terminal Gln or Asn could also be problematic. Finally, the most relevant positive selection criterion concerns the peptide's ionization efficiency and its MS/MS fragmentation properties. The [20−38], [44−65], and [102−122] peptides from the heavy chain of the mAb contained several unique residues, reflecting the variable-region heavy chain. These peptide sequences were searched against UniProt/Swissprot to exclude peptides present in the plasma proteome. A tryptic digest of the mAb was separated on a reversed-phase column, and tandem MS was performed on the [20−38], [44−65], and [102−122] peptides. Since the [102−122] peptide dissociated most efficiently, this peptide was selected for all subsequent MRM-MS experiments. The transitions for the doubly charged [102−122] peptide (m/z 1102) to three of its singly charged y-ions were used for the identification of the signature peptide. Two MRM transitions for the peptide were monitored to increase the selectivity of our quantification method ($[M + 2H]^{2+}$ 1102.20 → 580.30 (y_6^+-ion) and $[M + 2H]^{2+}$ 1102.20 → 780.30 (y_8^+-ion)) (Figure 1).

IS Labeling Strategy and IS Analysis by LC−MS/MS. The [102−122] peptide was used as a template for the IS. The labeling strategy was initiated to obtain a shift in mass of 20 Da between the signature peptide and the equivalent IS. Therefore, the four threonines of the signature peptide appear to be good candidates for the labeling.

The molecular weights of the heavy and light chains of the nonlabeled mAb (analyte) and labeled mAb (IS) obtained by MS analysis were consistent with the expected molecular weight depicted in Table 1. In addition, the LC−MS/MS analysis of the IS after trypsin digestion produced the expected mass shift on the equivalent labeled signature peptide (see Figure 3D). This indicated the incorporation of isotope-labeled threonine into the mAb. The two monitored transitions for the IS were $[M + 2H]^{2+}$ 1112.20 → 585.00 (y_6^+-ion) and $[M + 2H]^{2+}$ 1112.20 → 790.00 (y_8^+-ion).

Liquid Chromatography Optimization. For the present method, we wanted to keep the LC run short (<12 min) in order to increase the throughput of our assay. Neither nano nor capillary LC previously used for the quantification of proteins[13,21] was considered. During the method development, we found that signature peptide MS signal suppression by the matrix proved to be a very challenging analytical issue, which led to poor assay sensitivity. In our experiments, different columns were tested; these columns include an Agilent Zorbax C_{18} (30 mm × 2.1 mm, 1.8 μm particle), Agilent Zorbax Eclipse XD C_8 (50 mm × 2.1 mm, 3.5 μm particle), and ACE C_{18} (150 mm × 4.6 mm, 3.0 μm particle). The ACE column, under our LC conditions, was found to provide the highest sensitivity for the signature peptide with a signal-to-noise ratio enhancement by a factor of 6 compared to the Zorbax one (Figure 2). Interestingly, it is important to note that the higher length and wider diameter of the selected column will allow a better separation and higher loading capacity leading to a better assay reproducibility and sensitivity.

Assay Validation. The purpose of the present work was to validate our method in accordance to the FDA guidance for small molecules with regards to the dynamic range, precision, and accuracy. Several different batches of marmoset serum were extracted and analyzed using the LC−MS/MS assay described in the Experimental Section. No interference peaks were found at the retention time of the signature peptide in the chromatogram of a double-blank serum sample (Figure 3A). This indicated that the method is highly selective. In addition, the IS did not contribute to a signal in the mAb MRM channel (Figure 3B). In the chromatogram of a standard serum sample spiked with mAb (LLOQ 5.00 μg/mL) and IS (227 μg/mL) it can be noticed that the analyte is baseline-resolved from matrix endogenous com-

Table 5. Matrix Factor of Two Prepared Marmoset Sera with 5 and 500 μg/mL mAb

	mAb concn added in serum before extraction	
	5.00 μg/mL	500 μg/mL
	IS peak height after LC−MS/MS analysis	
serum digest, spiked with synthetic labeled signature peptide after extraction	10 082	8544
	11 626	9562
A: mean ($n = 2$)	10 854	9053
pure standard	19 069	19 069
	23 316	23 316
B: mean ($n = 2$)	21 193	21 193
matrix factor = A/B	0.51	0.43

Table 6. Absolute Recoveries Data for mAb and Signature Peptide after SPE

	mAb concn (μg/mL) spiked in serum		synthetic labeled IS concn (μg/mL)
	5.00	500.0	2.1
	synthetic labeled IS peak height after LC−MS/MS		synthetic labeled IS peak height after LC−MS/MS
serum spiked before extraction	7173	300 362	126 000
	5076	341 502	129 000
	5015	262 817	132 000
A: mean ($n = 3$)	5755	301 560	129 000
pure standard	44 162	2 260 183	212 000
	39 075	2 380 990	232 000
	37 962	2 359 432	220 000
B: mean ($n = 3$)	40 400	2 333 535	221 333
recovery = $A/B \times 100$	14%	13%	58%

pounds (Figure 3C). The chromatogram of the extracted serum sample (spiked with the IS) showed a baseline-resolved IS peak at the MRM channel of the IS. (Figure 3D). The calibration curves were linear over the concentration range of 5.00−1000 μg/mL of mAb with correlation coefficients (r^2) of duplicate calibration curves >0.99. The LLOQ for mAb determination in marmoset serum was 5.00 μg/mL, as defined by the lowest standard mAb concentration with accuracy within ±20% and a precision <20%. The intraday ($n = 5$) and interday ($n = 10$) precision and accuracy values summarized in Table 2 are within ±15% at all concentrations and within ±20% at the LLOQ.

The LC−MS/MS method was applied to the measurement of the mAb in marmoset serum samples after subcutaneous administrations of 50 mg/kg (one animal was considered at this dose level) and 150 mg/kg (three animals were considered at this dose level). Samples were measured in duplicate. The precision data of the two measured samples was found to be less than 15% for the majority of the duplicate analyses except for the concentrations below the LLOQ that were not reported (Tables 3 and 4) We can conclude that our method is precise. The calculated matrix effect value (ion suppression) obtained by MS on the signature peptide was around 0.5 (Table 5). This proves that the sample preparation procedure described in the earlier section was not optimal despite the use of an SPE cleanup step and a 150 mm length column for chromatographic separation. The sample cleanup performed after trypsin digestion is essential to reduce sample complexity. In a recent work, two purification steps have been applied to the sample purification.[17] The removal of the most abundant proteins with depletion kits[18,23] or by trapping the proteins of interest on immunoaffinity columns has been considered to reduce the sample complexity. For the quantification of mAbs, papain was used to deplete serum from larger proteins and enrich $F_{(ab)2}$ in the first step of the LC−MS/MS method.[24] Since the main purpose of this work was to demonstrate the usefulness of an isotope-labeled mAb as IS for the mAb quantification, no attempt was made on the sample cleanup improvement. The overall recovery of the mAb at two concentrations of 5.00 and 500 μg/mL ($n = 3$) was about 14%, and the one found after the sample purification on SPE was 58% (Table 6). The matrix effect factor was about 0.5. Taking these values into account, the losses of mAb or peptide produced during the digestion step was estimated at about 32%. Our data together with the recent data[13,14,21,22] published in the literature show that significant losses of proteins can occur during the sample workup. This strongly emphasizes the necessity to employ an IS right from the beginning.

Comparison between ELISA and LC−MS/MS for Marmoset PK Study of mAb. Serum samples obtained after subcutaneous administration of 150 mg/kg were both measured by LC−MS/MS and ELISA for the mAb concentration determination. The time profile graphs of the average concentration obtained by ELISA and LC−MS/MS are shown in Figure 4. The two curves displayed very similar profiles. However, compared to ELISA assay, LC−MS/MS showed about 1.6-fold higher exposure in terms of AUC and C_{max} (Table 7). To explain this discrepancy, we have artificially spiked in marmoset serum the mAb (50.0 μg/mL) with the antidrug at 100 μg/mL to check the trypsin digestion efficiency in the presence of anti-mAb−mAb complex. The data showed that approximately 80% of mAb was recovered from the complex compared to the digestion of free mAb at the same concentration (data not shown). Therefore, the difference between ELISA and LC−MS/MS (at least in the case reported here) can be explained by the fact that LC−MS/MS after

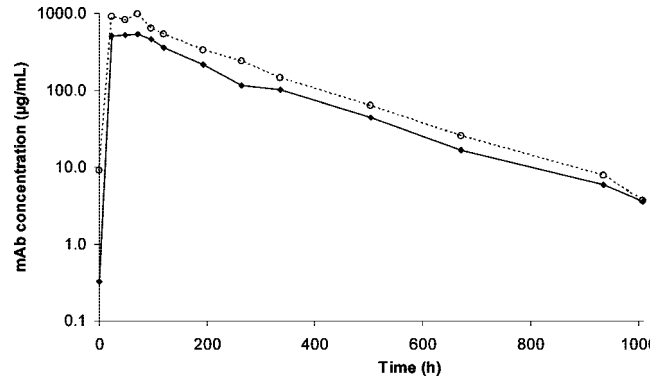

Figure 4. Comparison of mean concentration time profiles of mAb between (– – –) LC−MS/MS and (—) ELISA in marmoset serum.

(23) Chromy, B. A.; Gonzales, A. D.; Perkins, J.; Choi, M. W.; Corzett, M. H.; Chang, B. C.; Corzett, C. H.; Cutchen-Maloney, S. L. *J. Proteome Res.* **2004**, *3*, 1120–1127.

(24) Yan, B. X.; Valliere-Douglass, J.; Brady, L.; Steen, S.; Han, M.; Pace, D.; Elliott, S.; Yates, Z.; Han, Y. H.; Balland, A.; Wang, W. C.; Pettit, D. *J. Chromatogr., A* **2007**, *1164*, 153–161.

Table 7. Pharmacokinetic Parameters of mAb Obtained by ELISA and LC−MS/MS

parameters	units	ELISA	LC−MS/MS
dose	mg/kg	150	150
T_{max}	h	48−72	24
C_{max}	μg/mL	553	906
C_{max}/dose	(μg/mL)/(mg/kg)	3.69	6.04
AUC	μg·h/mL	113 502	181 443
AUC/dose	(μg·h/mL)/(mg/kg)	757	1210
AUC interval	h	0−1008	0−1008

digestion measured the total mAb in the samples, whereas ELISA can only measure the active or free mAb present in the sample.

CONCLUSIONS

A rapid, simple, and precise LC−MS/MS method was developed for the quantification of mAb using isotope-labeled mAb as IS. The method was successfully applied to the measurement of one of our mAb in marmoset serum to generate pharmacokinetic data in this species. Our data indicate that significant losses of proteins can occur during the sample workup. This strongly emphasizes the necessity to employ an IS right from the beginning. The challenge of future work will be to improve the assay sensitivity. In this respect, the use of different cleanup approaches or LC chromatography system such as ultraperformance liquid chromatography to reduce the sample complexity are some leads to explore.

ACKNOWLEDGMENT

The authors thank Axel Römer, A&M GmbH, Bergheim, Germany, for fruitful discussions on protein internal standards for protein analysis by LC−MS/MS, and further thank Francis Bitsch, Brian Granda, and Karen Wang from Novartis Institutes for Biomedical Research for sharing with us their knowledge on mAb determination by LC−MS/MS. We also thank Corinne Emotte, Marie-Rose Fischer, Monique Stefani, Daniela Parrizas, and Guy Taccard from DMPK Novartis Basel for providing technical support and for the critical review of this manuscript.

Received for review January 28, 2008. Accepted April 4, 2008.

AC800205S