

Immunopurification and Mass Spectrometric Quantification of the Active Form of a Chimeric Therapeutic Antibody in Human Serum

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In this study, we show that liquid chromatography coupled with tandem mass spectrometry provides a sensitive, specific, and accurate absolute quantification of Erbitux, a human:mouse chimeric mAb used for the treatment of colorectal cancer. Micrometric magnetized beads, functionalized with soluble epidermal growth factor receptor (sEGFR), the pharmacological target of Erbitux, were used for specific immunocapture of Erbitux allowing assessment of the antibody's biological potency and sample purification. Following digestion with trypsin, specific peptides from light and heavy chains were monitored in the selected reaction monitoring (SRM) mode. Assay variability below 20% was provided through optimization of the digestion step and rigorous monitoring of the whole analytical process using an appropriate internal standard. The 20 ng/mL lower limit of quantification was similar to that of ELISA methods. These results show that this mass spectrometric approach is a potential alternative for pharmacokinetic evaluation of mAbs during clinical development.

Targeted therapies using monoclonal antibodies (mAbs) have been used increasingly in recent years. Fourteen mAbs are currently on the market in various therapeutic areas, such as oncology, inflammation, infectious, and cardiovascular diseases. This number will likely increase in the years to come as more than 100 mAbs are currently in clinical development.¹

The preclinical and clinical developments of mAbs require accurate and specific bioanalytical methods for pharmacokinetic assessments. One analytical difficulty is that chimeric or humanized mAbs are similar to endogenous immunoglobulins, one of the most abundant proteins in plasma with concentrations around

10 mg/mL.² This is 1000- to 10 000-fold greater than that of circulating therapeutic antibodies and may represent a potential source of analytical interference. Assay development should also take into account structural modifications occurring during manufacturing or storage, such as deamidation, oxidation, and variable N-glycosylation. These modifications, associated with the in vivo elimination processes, may generate heterogeneity in the mAb structure that can affect its biological activity.^{3–5} Hence, the assay should be able to specifically determine the therapeutic active form of the mAb in the biological matrix.

Bioanalytical methods developed for the pharmacokinetic study of mAbs are mainly based on immunoassay approaches. Generally, after reaction with the therapeutic target bound on microtiter plates, the monoclonal antibodies are revealed by labeled anti-Fc antibody. These assays have high sensitivity but lack signal specificity, especially in human samples, since secondary antibodies may also detect nonspecifically bound endogenous antibodies. This interference leads to both matrix effects and selectivity issues, which require sample dilution and cause lack of sensitivity.⁶

Another approach would be the application of mass spectrometry methodologies and more particularly the use of electrospray (ESI) ionization allowing the coupling of liquid chromatography with tandem mass spectrometry (LC–ESI-MS/MS), which is a useful tool for drug quantification in biological fluids.⁷ The absolute quantification of proteins by LC–ESI-MS/MS in the selected reaction monitoring (SRM) mode can be carried out either on the intact protein or on specific peptides released by enzymatic digestion of the protein. Intact therapeutic protein quantification, without digestion, using LC–ESI-MS/MS is emerging and is applicable to pharmacokinetic studies with sufficient sensitivity and reproducibility.^{8–11} So far, the methods have only been applied

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to small proteins of molecular weight up to ~10 kDa. Extension to large proteins, such as mAbs, appears challenging. Protein characterized by high molecular weight (around 150 kDa) leads to complex ESI mass spectra with a huge number of charge states¹² which would lower sensitivity. Furthermore, acceptable chromatographic peak resolution for such large molecules implies the use of trifluoroacetic acid, which leads to mass spectrometric signal loss.¹³

An alternative strategy may consist of enzymatic digestion of the whole protein in the biological samples and SRM quantification of particular peptides. Whereas no application to mAbs has been published, a few examples of quantitative applications were recently presented for smaller proteins.^{14–17} This approach is, however, hampered by lack of sensitivity due to ionization suppression effects caused by the presence of endogenous species or tryptic peptides resulting from abundant proteins.¹⁸ A second potential disadvantage is that the biological potency of the antibodies cannot be assessed when only a peptide portion of the protein is monitored.

In order to circumvent some of these problems, immunocapture appears to be the most suitable isolation solution as previously described for proteins^{11,18–22} in biological matrixes. Here, we propose an immunocapture/mass spectrometry assay which targets Erbitux (Cetuximab), a mAb used for the treatment of colorectal cancer. This mAb (153 kDa) is a human:mouse chimeric antibody which specifically binds to the extracellular region of epidermal growth factor receptor (EGFR) and inhibits cellular proliferation in vitro and in vivo.²³ The assay was designed to selectively measure the active form of Erbitux. An immunocapture step employing soluble epidermal growth factor receptor (sEGFR), the Erbitux target, was set up to extract and concentrate the mAb from human serum. The antibody is then chemically released, enzymatically digested, and analyzed by LC–ESI-MS/MS through monitoring of specific peptides. The use of an appropriate internal standard which monitors the whole analytical

process enables sensitive and accurate quantification of Erbitux in human serum.

MATERIALS AND METHODS

Chemicals and Reagents. Erbitux and sEGFR were obtained from Merck KGaA (Darmstadt, Germany) as aqueous 2 mg/mL solutions. The mouse monoclonal antibody to human EGFR was from R&D Systems (Minneapolis, MN). Sequencing grade modified trypsin was from Promega (Madison, WI). A 15-mer synthetic peptide corresponding to the light chain of the therapeutic antibody tryptic fragment (LT3, ASQSIGTNIHWYQQR) was synthesized by Genepep (Montpellier, France), and two other tryptic fragment of the heavy chain (HT4, GLEWLGVWSGGNT-DYNTPFSTR, and HT23, VVSVLTVLHQDWLNGK) were synthesized in-house using a Applied Biosystems biosynthesizer. Human serum from healthy subjects was obtained from Biopredic (Rennes, France). Gradient grade methanol from Merck (Darmstadt, Germany), HPLC quality acetonitrile from SDS (Peypin, France), and analytical formic acid from Sigma (Sigma Chemical Co., St. Louis, MO) were the solvents used. Tosylactivated magnetic beads (Dynabeads M-280) were from Invitrogen (Carlsbad, CA). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Sample Preparation. Calibration standards were prepared by diluting Erbitux in pooled human serum to 2, 1, 0.5, 0.2, 0.1, 0.05, and 0.02 $\mu\text{g/mL}$. The 2 $\mu\text{g/mL}$ (13 nM) calibration standard was prepared by spiking 2 mL of human serum with 40 μL of a 100 $\mu\text{g/mL}$ solution in Tris buffer. The other calibration standards were prepared by serial dilution in human serum. Quality control samples (QCs) were used to evaluate the accuracy and precision during the validation and were prepared in the same way as calibration standards at 1.5, 0.4, 0.06, and 0.02 $\mu\text{g/mL}$. The 1.5 $\mu\text{g/mL}$ QC was prepared by adding 45 μL of a 100 $\mu\text{g/mL}$ solution of Erbitux to 3 mL of serum, and the other QCs were prepared by serial dilution. A mouse monoclonal antibody to human EGFR used as internal standard (IS) was added to calibration standard and QCs. An amount of 10 μL of 25 $\mu\text{g/mL}$ mouse antibody in Tris buffer was added to 500 μL of serum to give a final concentration of 0.5 $\mu\text{g/mL}$. Aliquots of 500 μL were used for analysis. All samples were submitted to the immunocapture and digestion steps prior to LC–MS/MS analysis.

Immunocapture and Digestion. M-280 tosylactivated magnetic beads (30 μL suspension equivalent to 0.9 mg of beads per 500 μL of serum sample) were washed with 500 μL of borate buffer (0.1 M, pH 9.5) following the protocol provided by the supplier. The beads were separated from solution using a magnetic particle concentrator for microcentrifuge tubes (Dynal MPC-S, Dynal Biotech, Oslo, Norway). After removal of the washing solution, the beads were incubated for 4 h at room temperature with gentle shaking with sEGFR (diluted to 100 $\mu\text{g/mL}$ in borate buffer) at a ratio of 3 μg of protein per mg of beads. sEGFR was covalently bound to the beads by reaction of the protein amino groups with the beads' tosylate groups. Following incubation, noncovalently linked sEGFR was removed by washing two times with PBS buffer (pH 7.4, 0.1% BSA), and the magnetic beads were resuspended in Tris buffer (0.2 M, pH 8.5, 0.1% BSA) and incubated overnight at 4 °C to block free tosylate groups. The beads were washed with PBS buffer (pH 7.4, 0.1% BSA) and then resuspended in Tris-

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HCl (50 mM, pH 8.5, 100 $\mu\text{g/mL}$ BSA) at a concentration of 90 mg of beads per mL.

Functionalized beads (0.9 mg in 10 μL) were added to serum samples containing Erbitux and IS for immunocapture, and incubation was performed at room temperature with gentle rotation for 2 h. After washing twice with 500 μL of Tris-HCl (50 mM, pH 8.5, 100 $\mu\text{g/mL}$ BSA) to remove weak nonspecific binding, Erbitux and IS were eluted under acidic conditions with 50 μL of Tris-HCl (50 mM, pH 2, 100 $\mu\text{g/mL}$ BSA) for 15 min while vortexing.

Whole supernatant (50 μL) was transferred for denaturation and enzymatic digestion. Reduction was performed by adding to the supernatant 10 μL of basic dithiothreitol (10 mM in 250 mM NaOH) to ensure pH 8.5 after addition of this reducing buffer and to optimize enzyme activity. After 30 min at 60 $^{\circ}\text{C}$ to denature the antibodies, 0.1 μg (5 μL) of trypsin was added. Samples were then incubated for 3 h at 40 $^{\circ}\text{C}$, and methanol with 10% formic acid (10 μL) was added at the end of the reaction. Methanol was used to avoid nonspecific binding of tryptic peptides. Samples were transferred into an HPLC vial and injected (40 μL) into the chromatographic system.

Qualitative Mass Spectrometry for Tryptic Peptide Identification. Peptide identification, before selection of the best candidates for the quantification of Erbitux, was performed using a combination of nanoLC-TOF-MS, nanoLC-QTOF-MS/MS, and MALDI-TOF-MS.

Mass spectrometric detection of tryptic digests was performed with an Ultimate 3000 nanoLC (Dionex, Sunnyvale, CA) coupled with a QTOF-I (Waters, Milford, MA) equipped with a nanoelectrospray ion source and a PicoTip Emitter (FS360-20-10-D20-C7, New Objective Inc., Woburn, MA). Erbitux or mouse monoclonal antibody digest samples (5 μL at 100 $\mu\text{g/mL}$) were stacked on a C trap cartridge and eluted on a reversed-phase column (C18 Pepmap, 75 μm i.d. $\times$ 150 mm, 3 μm particle size, 100 $\text{\AA}$). Elution was performed at a flow rate of 200 nL/min with water containing 0.1% (v/v) formic acid as eluent A and acetonitrile containing 0.1% (v/v) formic acid as eluent B. The analytical gradient profile was as follows (min/% of B in A): 0/0, 1/0, 50/70, 52/100, 60/100, 62/0, 75/0. The column was thermostated at 30 $^{\circ}\text{C}$. Ionization on the QTOF-I was performed in the positive mode using a capillary voltage of 2000 V; the cone voltage was set to 25 V. Mass calibration was done externally, and the cycle time was 1 and 3 s in the MS and in the MS/MS experiments, respectively. Data were acquired by the TOF analyzer from m/z 200 to 2500. Doubly and triply charged ions of interest obtained in TOF MS were selected in the quadrupole for fragmentation in the hexapole of the QTOF with an optimized collision energy of $E_{\text{lab}} = 30$ and 16 eV, respectively. Argon was used as the collision gas.

MALDI-TOF-MS analyses were performed on a Voyager DE-STR instrument (Applied Biosystems, Les Ulis, France) equipped with a pulsed nitrogen laser (337 nm). Samples were cocrystallized with α -cyano-4-hydroxycinnamic acid and analyzed in the positive mode using the reflector and the extraction delay at 140 ns for higher resolution. The accelerating voltage was set to 20 000 V. All mass spectra were averaged from 500 laser shots at a repetition rate of 20 Hz. Internal calibration of the mass spectra was done by postacquisition with trypsin autolysis products at m/z 842.51 and 2211.10.

Table 1. Transitions Selected for Quantification of Triply and Doubly Protonated Peptides, the Corresponding Cone Voltage Values at Optimized Collision Energy of the LT3, HT4, HT23, and IS^a Peptides Are Reported

peptide		transition	cone voltage (V)	collision energy (eV)
LT3	(3+)	597.3 $\rightarrow$ 652.1	25	15
	(2+)	895.1 $\rightarrow$ 1303.1	36	34
HT4	(3+)	857.5 $\rightarrow$ 908.7	28	15
	(2+)	1286.2 $\rightarrow$ 1703.1	40	34
HT23	(3+)	603.8 $\rightarrow$ 805.8	28	15
	(2+)	904.8 $\rightarrow$ 997.8	40	32
IS ^a	(3+)	603.7 $\rightarrow$ 766.4	25	15

^a IS: internal standard.

Quantitative Mass Spectrometry. Quantitation of Erbitux was performed on a triple-quadrupole mass spectrometer, Quattro LCZ (Waters, Manchester, U.K.) coupled to an HPLC Alliance. Chromatography was performed on a C18 guard column followed by a Zorbax SB-C18 column (150 mm $\times$ 2.1 mm i.d., 5 μm particle size, 80 $\text{\AA}$ porosity). The column was eluted at a flow rate of 0.3 mL/min using as mobile phase A water/butanol (99/1, v/v) and as mobile phase B acetonitrile, both containing 0.1% (v/v) formic acid. The analytical gradient profile was as follows (min/% of mobile phase B in A): 0.0/5, 4.0/25, 12.0/37, 12.1/100, 13.0/100, 13.1/5, and 17.0/5. The column temperature was 50 $^{\circ}\text{C}$, and the injection volume was 40 μL . A mouse monoclonal antibody was immunoprecipitated and digested as Erbitux. One peptide from the variable region of the mouse antibody used as the internal standard was coeluted with LT3 (tryptic peptide from the light chain, see the Results section) and was then used as a surrogate measure of IS. Under these conditions, the retention time was 5.9 min for LT3 (tryptic peptide from the light chain) and IS, 9.1 min for HT23, and 11.6 min for HT4 (tryptic peptides from the heavy chain, see the Results section).

The HPLC column was coupled to the API source of the tandem mass spectrometer via an electrospray interface. Nitrogen was used as the nebulizing gas (115 L h^{-1}) and drying gas (900 L h^{-1}). The ESI source was operated in the positive ion mode using 3500 V as the high voltage for ionization, and the produced protonated molecular species were selected for low-energy collision processes in MS/MS experiments in the SRM acquisition mode. Collision-induced dissociations (CID) were achieved with argon at a pressure of 2.2×10^{-3} mbar. Two SRM transitions were performed from each peptide as reported in Table 1. The dwell-time for each SRM transition was 0.2 s. The two reconstituted chromatograms obtained for each peptide were summed for integration to increase signal-to-noise ratio and to take into account possible variability of charge state distribution according to the source conditions. Moreover, it was checked that the ratio of the two SRM transitions for each peptide was constant in each run. To maximize sensitivity, the cone voltage and collision energy were optimized for each transition (Table 1). Other parameters were optimized by LT3 peptide infusion. Source block temperature was set to 100 $^{\circ}\text{C}$ and drying temperature to 350 $^{\circ}\text{C}$. Measurement of the therapeutic antibody concentration in serum was based on quantification of the LT3 fragment. The HT4 over LT3 area ratio was also measured. HT4 was used in this study as a confirmation

peptide. HT23 was also monitored to evaluate nonspecific binding for each sample.

Quantification of Erbitux by the LC–MS/MS Method in Spiked Human Serum. Calibration curves were established as a quadratic regression function after 1/X weighting of the LT3/IS peak area ratio versus Erbitux concentration of the seven standard samples (from 0.02 to 2 $\mu\text{g/mL}$).

Accuracy and precision of the method were evaluated during 3 days by analyzing the four QC samples (0.02, 0.06, 0.4, and 1.5 $\mu\text{g/mL}$) in four replicates. Intra- and interday assay accuracy (ratio of the measured concentration to the nominal concentration multiplied by 100) and precision (percent coefficient of variation, % CV) were calculated at each concentration.

The recovery of the immunocapture step and the stability in the autosampler was estimated as follow. Two sets of 10-fold more concentrated solutions were prepared in elution buffer at 20, 10, 5, 2, 1, 0.5, and 0.2 $\mu\text{g/mL}$. These samples were submitted not to immunocapture but directly to denaturation and enzymatic digestion. This 10-fold concentration factor was used since the volume of the samples in serum was 500 μL and the elution volume after immunocapture was 50 μL . The signal of the immunocapture samples in serum was compared to the signal of the concentrated solutions in elution buffer. One set was analyzed by LC–ESI-MS/MS first, before the immunoextracted samples, and the second set was analyzed at the end of the run in the same conditions to check the stability of tryptic products in the autosampler. The first curve obtained by plotting the first set of control samples should superimpose on the curve of the second set to match stability criteria. Efficiency of trypsinization was also monitored using these control samples.

RESULTS AND DISCUSSION

Selection of Peptides for Quantification. The strategy for quantification involved the selection of tryptic peptides representative and specific to the variable regions of both the light and heavy chains. The corresponding sequences were submitted to similarity searches (BLAST) to ensure detection specificity. These different peptides were monitored by LC–ESI-MS/MS.

The first step of the development was to identify specific peptides with the most intense signal in mass spectrometry, among those produced by digestion with trypsin after reduction with dithiothreitol. Their molecular weights were determined by both the nanoLC–ESI-TOF-MS and MALDI-TOF-MS techniques and were compared with predicted peptides from theoretical digestion (Mass Lynx version 4.0) (59 expected peptides). Both techniques were used since they gave complementary information for peptide identification owing to their different ionization potencies. On the one hand, peptides identified by MALDI-TOF-MS (31 peptides) covered 88% of the sequence of the light chain and 65% of the heavy chain. Some peptides were not detected (8% of the light chain and 22% of the heavy chain) since they were outside the acquisition m/z range (500–5000 amu) used during the MALDI-TOF-MS experiments. They were denoted from LT1 to LT20 for the light chain and from HT1 to HT39 for the heavy chain, as reported in Table 2. The peaks from the mass spectrum were assigned to tryptic peptides by matching measured to expected molecular weights with less than 40 ppm of mass accuracy. On the other hand, nanoLC–QTOF-MS experiments provided 83% of sequence coverage of the light chain and 56% of

the heavy chain for a total of 31 peptides identified. It should be noted that the amino acid sequence of Erbitux contains three potential N-glycosylation sites based on the presence of the N–X–S/T–Y consensus sequence, i.e., LT4, HT8, and HT22 peptides. Proline at X or Y is highly unfavorable to glycosylation,²⁴ which explains the detection of the LT4 peptides containing the NGSP sequence (see Table 2 for peptides sequence). It has recently been demonstrated that the two potential sites in the heavy chains are glycosylated,²⁵ which could explain the absence of HT8 and HT22 as nonglycosylated peptides.

Peptides identified by both the ESI-TOF-MS and MALDI-TOF-MS experiments were kept for potential use in quantification. ESI-TOF-MS and MALDI-TOF-MS gave overlapping information since 27 peptides (out of the 59 theoretical tryptic peptides, 62% sequence coverage) were identified by both techniques. First, 31 peptides were identified by ESI-TOF-MS (65% sequence coverage), and then 31 were identified by MALDI-TOF-MS (73% sequence coverage). Two high molecular weight peptides identified only by MALDI-TOF-MS explain this significant increase in sequence coverage. The MALDI-TOF mass spectrum obtained after reduction and digestion of Erbitux with trypsin is shown in Figure 1.

Since Erbitux is a human:mouse chimeric mAb, its Fc fragment is identical to human IgG1, for which the concentration in human plasma is around 10 mg/mL. To reach the required sensitivity, a unique peptide sequence is a key prerequisite to avoid interference with human IgG₁. Hence, for mAb quantification, specificity criteria can be met by selecting peptides in variable regions located in the Fab fragments. The Erbitux Fab fragment binds exclusively to domain III of sEGFR, and both heavy and light chains of the antibody are involved. Nine amino acids located within the LT3, LT6, LT7, HT4, and HT9 peptides are involved²³ in this binding. Among these potential candidates for quantification, those without miscleavage and showing higher desorption and ionization efficiency were selected. This led to the choice of one peptide from the light chain (LT3) and another from the heavy chain (HT4). Although the LT3 peptide contains a Trp residue that might be sensitive to oxidation, we have never observed the corresponding peptide bearing an oxidized Trp residue even after prolonged storage. To estimate nonspecific binding of endogenous human IgG to magnetic beads during the immunocapture step, a third tryptic peptide (HT 23) of the Fc region was monitored, chosen for its high ionization efficiency. NanoLC–QTOF-MS/MS spectra of these three peptides are shown in Figure 2.

Optimization of Trypsin Digestion. Digestion had to be carefully optimized since many factors, such as pH, ionic strength, temperature, and incubation time, can influence the efficiency of proteolysis. We chose Promega trypsin, which exhibits limited self-digestion and retains its activity over a long period. With previous work in mind,²⁶ the optimal protein digestion was obtained at pH 8.5 in a 50 mM Tris-HCl buffer with 1 mM CaCl_2 and 100 $\mu\text{g/mL}$ BSA at a temperature of 40 °C with 0.1 μg of trypsin.

In order to decrease the number of steps in the analytical process, the need for protein denaturation, reduction, and alkyl-

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Table 2. Tryptic Peptides from Erbitux Identified by MALDI-TOF-MS and NanoLC-ESI-QTOF-MS^a

fragment	sequence	calculated mass	experimental mass	
			MALDI-TOF	ESI-QTOF
HT1 (1–5)	pQVQLK	597.35	597.35	597.34
HT2 (6–38)	QSGPGLVQPSQSLSTCTVSGFSLTNYGVHWVR	3504.75	3504.62	
HT3 (39–43)	QSPGK	515.27		
HT4 (44–66)	GLEWLGVWSSGNTDYNTPFTSR	2569.22	2569.15	2569.38
HT5 (67–71)	LSINK	573.35		
HT6 (72–75)	DNSK	462.21		
HT7 (76–81)	SQVFFK	754.40	754.40	754.42
HT8 (82–97)	MNSLQSNDAIYYCAR	1848.81		
HT9 (98–123)	ALTYDYEFAYWGQGLVTVSAATK	2904.39	2904.37	2904.88
HT10 (124–135)	GPSVFPLAPSSK	1185.64	1185.63	1185.58
HT11 (136–149)	STSGGTAALGCLVK	1263.65	1263.64	1263.66
HT12 (150–212)	DYFPEPVTWSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHK (PSNTK)	6655.29		
HT13 (213–215)	VDK	360.20		
HT13+14 (213–216)	VDKR	516.30		516.23
HT15 (217–220)	VEPK	471.27		
HT16 (221–224)	SCDK	451.17		
HT17 (225–250)	THTCPPCPAPELLGGPSVFLFPPK (PK)	2729.41	2729.36	
HT18 (251–257)	DTLMISR	834.43	834.43	834.46
HT19 (258–276)	TPEVTCVVVDVSHEDPEVK	2081.00	2080.97	2081.10
HT20 (277–290)	FNWYVDGVEVHNAK	1676.79	1676.77	1676.85
HT21 (291–294)	TK (PR)	500.31		500.19
HT22 (295–303)	EEQYNSTYR	1188.50		
HT23 (304–319)	VVSVLTVLHQDWLNGK	1807.00	1806.96	1806.95
HT24 (320–322)	EYK	438.21		
HT25 (323–324)	CK	249.11		
HT26 (325–328)	VSNK	446.25		
HT27 (329–336)	ALPAPIEK	837.50	837.49	837.54
HT28 (337–340)	TISK	447.27		
HT29 (341–342)	AK	217.14		
HT30 (343–346)	GQPR	456.24		
HT31 (347–357)	EPQYITLPPSR	1285.67	1285.66	1285.72
HT32 (358–362)	DELTK	604.31		604.35
HT33 (363–372)	NQVSLTCLVK	1103.60	1103.60	1103.46
HT34 (373–394)	GFYPSDIAVEWESNGQPENNYK	2543.12	2543.10	2543.44
HT35 (395–411)	TTPPVLDSDGSFFLYSK	1872.91	1872.89	1872.94
HT36 (412–416)	LTVDK	574.33		574.30
HT37 (417–418)	SR	261.14		
HT38 (419–441)	WQQGNVFCSCVMHEALHNHYTQK	2743.24	2743.17	2743.29
HT39 (442–449)	SLSLSPGK	787.44		
LT1 (1–18)	DILLTQSPVILSVSPGER	1923.07	1923.06	1923.11
LT2 (19–24)	VSFSCR	697.32	697.32	
LT3 (25–39)	ASQSIGTNIHWYQQR	1787.87	1787.87	1787.77
LT4 (40–45)	TNGSPR	630.31	630.31	
LT5 (46–49)	LLIK	485.36		
LT6 (50–61)	YASESISGIPSR	1265.63	1265.62	1265.56
LT7 (62–103)	FSGSGSGTFTLSINSVESEDIADYYCQQNNWNP TTFGAGTK	4507.97	4508.00	4507.36
LT8 (104–107)	LELK	501.32		
LT8+9 (104–108)	LELKR	657.42		
LT10 (109–126)	TVAAPSVFIFPPSDEQLK	1945.02	1944.99	1945.13
LT11 (127–142)	SGTASVVCLLNNFYP	1739.87	1739.87	1739.96
LT12 (143–145)	EAK	346.19		
LT13 (146–149)	VQWK	559.31	559.32	559.31
LT14 (150–169)	VDNALQSGNSQESVTEQDSK	2134.96	2134.93	2135.08
LT15 (170–183)	DSTYSLSTLTLSK	1501.75	1501.73	1501.84
LT16 (170–183)	ADYEK	624.28		
LT17 (170–183)	HK	283.16		
LT18 (191–207)	VYACEVTHQGLSPVTK	1817.90	1817.86	1817.95
LT19 (208–211)	SFNR	522.26	522.26	522.27
LT20 (212–213)	GA	146.07		

^a The peaks from the mass spectrum were assigned to tryptic peptides by matching measured to expected molecular weights with a 40 ppm mass accuracy. Peptides were denoted from HT1 to HT39 for the heavy chain and from LT1 to LT20 for the light chain

ation was investigated. Protein denaturation is required to break tertiary structures of the protein and to increase accessibility of

trypsin to its cleavage sites. Temperature, dithiothreitol, and 8 M urea were tested as denaturing agents. These experiments led to

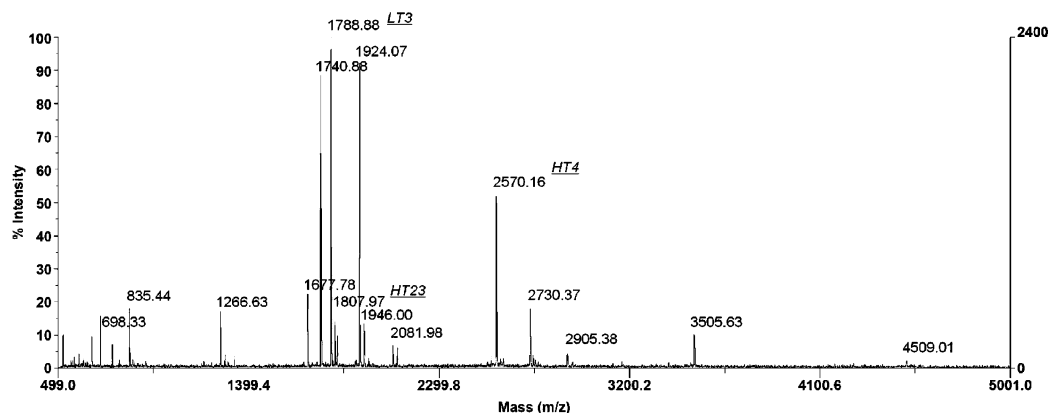


Figure 1. MALDI-TOF mass spectrum obtained after reduction and enzymatic digestion of mAb Erbitux with trypsin. Monitored peptides for quantification (LT3 at m/z 1788.88 and HT4 at m/z 2570.16) and for nonspecific binding estimation (HT23 at m/z 1807.97) are indicated.

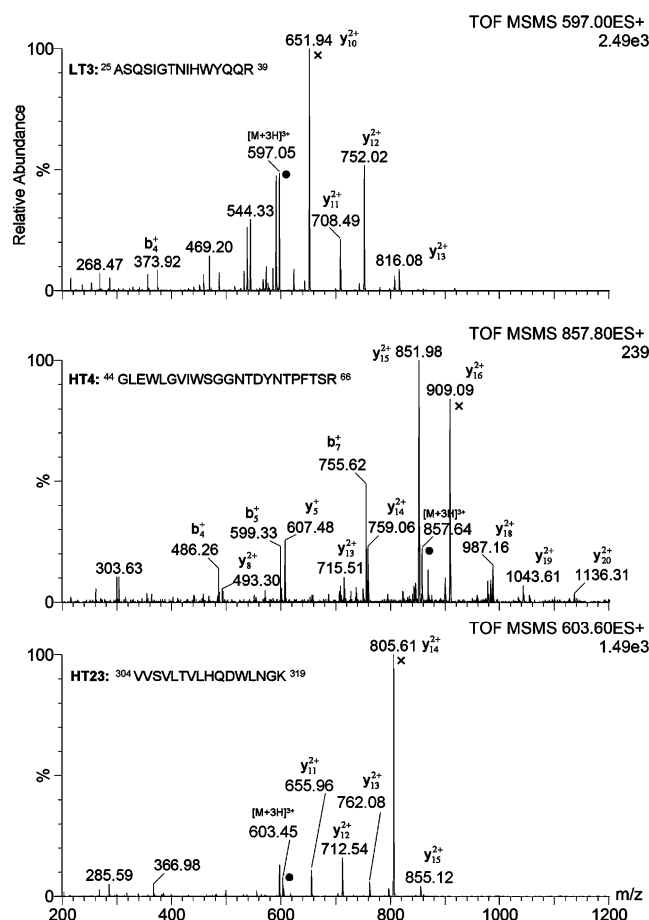


Figure 2. Product ion spectra of the triply charged tryptic peptides LT3 at m/z 597.00, HT4 at m/z 857.80, and HT23 at m/z 603.60, obtained by nanoLC-ESI-QTOF-MS/MS. Parent ion masses and selected daughter ions for SRM transitions are labeled, respectively, by ● and ×. The amino acid sequence of the parent ion peptides is indicated.

the conclusion that addition of dithiothreitol to a final concentration of 1.5 mM combined with heating at 60 °C for 30 min leads to effective denaturation. Urea addition did not further improve the peptide signal and was therefore not used during denaturation (data not shown).

A second treatment commonly used in proteomics is derivatization of cysteines with alkylating agents.²⁷ We compared the signals obtained without a derivatization step and with an

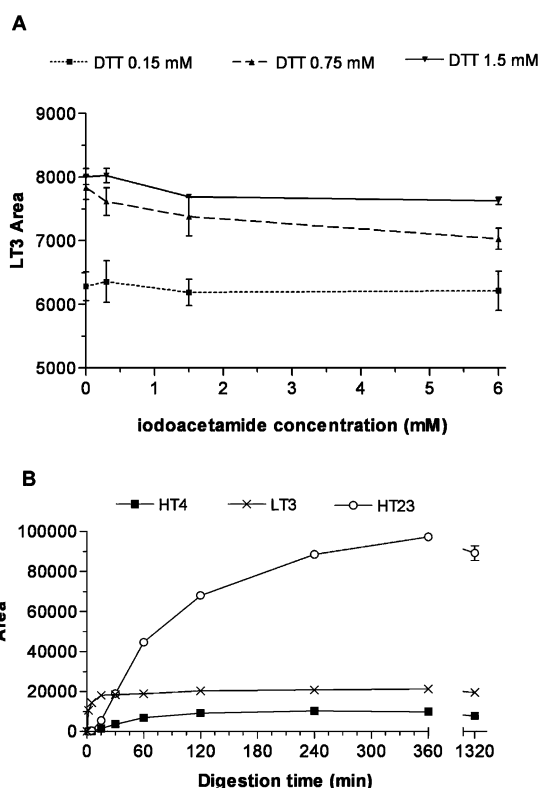


Figure 3. (A) LT3 formation with increased concentration of iodoacetamide (0.3, 1.5, 6 mM final concentration) and DTT (0.15, 0.75, 1.5 mM). (B) Kinetics of Erbitux (at 20 $\mu\text{g/mL}$) digestion, through measurement of peptides LT3, HT4, and HT23 formation between 0 and 22 h with no derivatization step. Digestion is performed in 50 mM Tris-HCl buffer with 1 mM CaCl_2 and 100 $\mu\text{g/mL}$ BSA at 40 °C.

increased concentration of iodoacetamide. No signal increase was obtained for HT4 and HT23, whereas a slight decrease in peak intensity was observed for LT3 (Figure 3a). No derivatization was finally performed in the assay.

The kinetics of the Erbitux peptide formation (LT3, HT4, and HT23) were then assessed after stopping the digestion at various incubation times with 10 μL of methanol containing 10% formic acid, which lowered the pH to 2. The efficiency of this stop solution was first investigated. Before the addition of trypsin, 10 μL of

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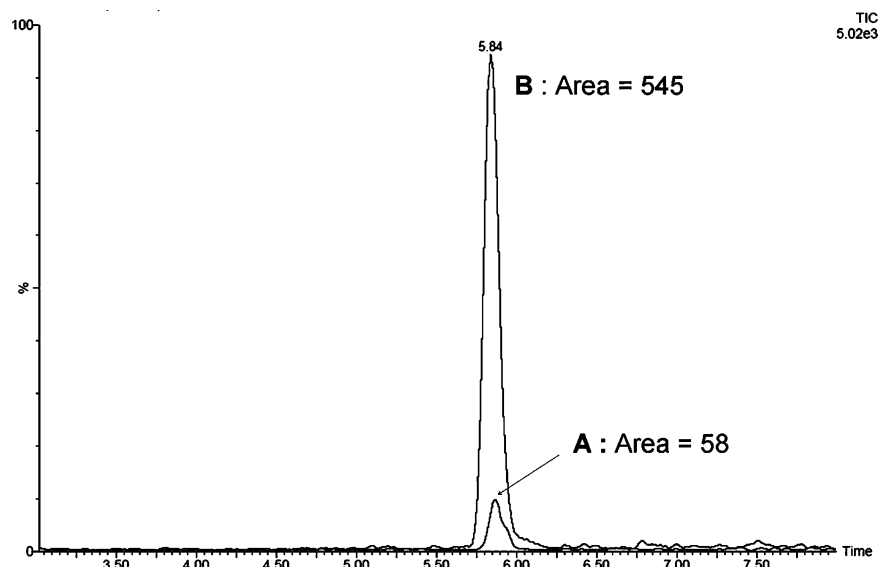


Figure 4. Reconstituted LT3 signals after trypsin digestion from (A) a sample of Tris-HCl 50 mM buffer (1 mM CaCl_2 and 100 $\mu\text{g/mL}$ BSA) spiked with 200 ng/mL Erbitux and (B) a sample of human serum spiked with 200 ng/mL Erbitux submitted to immunocapture. Chromatographic separation was performed with a gradient mobile phase as described in the experimental section.

methanol (10% formic acid) was added to a sample of denatured and reduced Erbitux. This acidified sample was then incubated for 4 h at 40 °C, and no tryptic peptide was observed. Then, the kinetic profiles of the three peptides were determined as shown in Figure 3b, with a 20 $\mu\text{g/mL}$ Erbitux solution. A time of 15 min is sufficient to reach a plateau for LT3, whereas formation of the two other peptides is slower, as 2 h is required for HT4 and at least 6 h for HT23. A 3-h incubation was subsequently used for quantification of the two specific peptides (LT3 and HT4). A 100% release of HT23 upon digestion was not considered as mandatory to shorten the analytical process. Moreover, this peptide was not used for quantification, and the variability induced by this kinetic interruption is far below variability induced by nonspecific binding. In conclusion to this digestion step optimization, maximum intensity was obtained with a denaturation step at 60 °C under reducing conditions (1.5 mM DTT final concentration) before addition of 5 μL of trypsin (0.1 μg) followed by a 3-h incubation at 40 °C.

Immunocapture of Erbitux. Sample preparation for LC–MS/MS experiments allows concentration of the analyte and removal of components that reduce ionization efficiency. Traditional methods rely on solid-phase extraction or protein precipitation. These approaches are suitable for polypeptides and small proteins^{8,9,28} but are not sufficiently selective or are incompatible with larger proteins such as antibodies.

Here, we selected immunocapture to measure specifically the antibody form that binds its pharmacological target and thus constitutes the most selective purification of the active mAb from other IgG. For ease of assay development and as described previously,²⁹ we used magnetic beads that were covalently linked to sEGFR. The immunocapture was performed on 500 μL of plasma, a volume which is compatible with usual blood sampling during human pharmacokinetic studies.

Erbitux can be eluted from the beads using organic solvents, high ionic strength solutions, or low pH solutions. Elution was developed keeping in mind that the buffer must be compatible with the optimized conditions for digestion with trypsin. Tris buffer at pH 2 was used for elution, and the pH was raised to ~ 8.5 by NaOH addition prior to trypsin digestion. Addition of organic solvent, i.e., methanol and acetonitrile, was tested but not used since a decrease in Erbitux peptide signal was observed, especially when the percentage of organic solvent was increased. The main hypothesis is that the mAb is partially precipitated under these experimental conditions.

Finally, a 10-fold increase in sensitivity was obtained thanks to this immunocapture step (Figure 4) since the elution volume was set at 50 μL .

Internal Standard and Calibration Curves. The use of an internal standard (IS) is required for accurate and reproducible quantification, especially when sample preparation involves numerous steps. Although tryptic peptides labeled with stable isotopes are often used,^{30–32} this does not allow for checking of the sample preparation steps, such as immunocapture and enzymatic digestion. A more suitable approach consists in the introduction of stable isotope residues in the whole protein backbone and the further monitoring of one (or more) labeled tryptic peptides.³³ Although this has already been applied to several proteins (RK5,⁸ ADH,¹⁷ insulin¹⁰), the production of labeled proteins is a highly complex and time-consuming process.

Surrogate internal standards, such as analogue proteins from other species³⁴ and recombinant proteins differing by a few amino acids²² or chemical derivatization,¹¹ have been reported. In the present method, we chose another alternative by monitoring the

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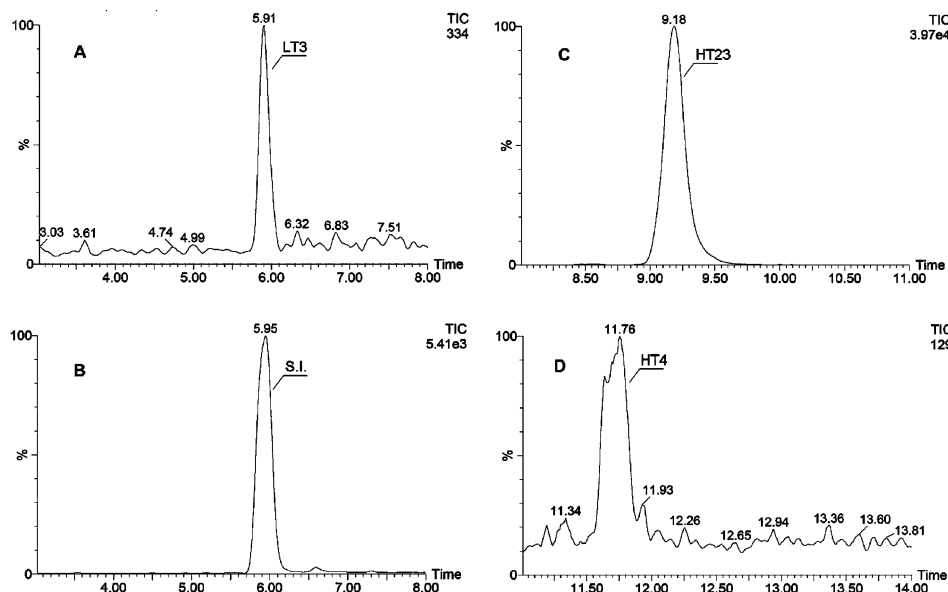


Figure 5. SRM transitions of (A) LT3 ($[M + 3H]^{3+}$ at m/z 597.3 and $[M + 2H]^{2+}$ at m/z 895.1), (C) HT23 ($[M + 3H]^{3+}$ at m/z 603.8 and $[M + 2H]^{2+}$ at m/z 904.8) and (D) HT4 ($[M + 3H]^{3+}$ at m/z 857.5 and $[M + 2H]^{2+}$ at m/z 1286.2) peptides at an Erbitux concentration of 20 ng/mL (LLOQ), after immunocapture in human serum and digestion with trypsin. (B) Internal standard ($[M + 3H]^{3+}$ at m/z 603.7) was added at 500 ng/mL. Separation conditions are reported in the Materials and Methods section.

whole analytical process, especially the immunocapture step. This internal standard consists of a commercial mouse monoclonal antibody specific to human sEGFR. Assuming that such an immunoglobulin structure will behave similarly to Erbitux during the enzymatic digestion, specific tryptic peptides can be selected as described previously for Erbitux and further used for the LT3 area standardization. A peptide from the variable region of the IgG light chain with the same retention time as LT3 was chosen to monitor the IS concentration. Erbitux quantification is then performed by measuring LT3 over the IS peptide area ratio. In addition, the area ratio of HT4 over LT3 was determined to enhance confidence in the Erbitux determination. The SRM transitions of HT4 was used in this study as a surrogate measure of the heavy chain (Figure 5).

In the initial steps of the immunocapture development, we found that the standard curve determined directly in the elution buffer (Tris-HCl) presented an exponential response with Erbitux concentrations. We found that this was due to nonspecific adsorption, most probably of the intact Erbitux rather than tryptic peptides. To circumvent this problem, we added bovine serum albumin (BSA) to the buffer solution before the enzymatic digestion. Since high quantities of BSA caused ion suppression, we selected the concentration of 100 $\mu\text{g/mL}$ for which the maximum response of LT3 was obtained. Under these conditions, the linearity of the response was obtained in elution buffer between 0.2 and 20 $\mu\text{g/mL}$ (Figure 6a) without immunocapture.

The calibration curve obtained after immunopurification of Erbitux from serum samples is shown in Figure 6b and ranged between 20 and 2000 ng/mL, consistent with the 10-fold increase in sensitivity. The tendency of nonlinearity observed at high Erbitux concentrations was related to the saturation of binding sites, i.e., sEGFR coated on the beads. A 2-fold increase in the amount of functionalized beads was not sufficient to change this behavior significantly. In order to obtain a higher linear range of the calibration curve, a superior amount of beads would be

required. However this will not be compatible with a low elution volume. Nevertheless, the amount of sEGFR coated allows a quantification range of 2 logarithms. The HT4 over LT3 area ratio was constant over the whole range of Erbitux concentrations, as described in Figure 6c. The mean area ratio was observed at 0.75, $\text{CV} = 8\%$, which implies that the ratio of these two peptides, LT3 and HT4, that belong, respectively, to the light and to the heavy chain, can be measured with an acceptable robustness.

Method Applicability to Human Serum. Selectivity was first checked, for peptides LT3, HT4, and HT23, with pooled blank human serum from different individuals. No signal was found at the retention time and SRM transitions of LT3 and HT4. However, in the absence of Erbitux, a significant response was observed for the SRM transitions of HT23 with a signal roughly equivalent to an immunoglobulin concentration of 2 $\mu\text{g/mL}$, this corresponds to the nonspecific binding of IgG to the beads. This effect, shown in Figure 5c at the LLOQ, keeps one from performing a reliable quantification using such a peptide located in the constant region of mAb. This result highlights the need to monitor peptides from the variable domains of the mAb for accurate quantification of low concentrations of therapeutic antibodies.

In order to determine the extraction recovery, we compared the signals from control solutions and in spiked serum by means of the area ratio of LT3 and internal standard. Overall extraction recovery was between 45% and 100% for Erbitux, with a lower percentage at the highest Erbitux concentration. A constant recovery of 45% ($\text{CV} = 9\%$) over the whole Erbitux concentration range was observed for the internal standard.

Finally, spiked QC samples in human serum at four concentrations were analyzed repeatedly over 3 days for determination of intra- and inter-run precision of the assay. Above the lower limit of quantification (LLOQ), CV% were between 6% and 17% for intra-assay ($n = 4$) and between 9% and 18% for inter-assay precision ($n = 12$ over 3 days). Accuracy was between 85% and 105%. The LLOQ was determined to be at 20 ng/mL. At this level, intra- and

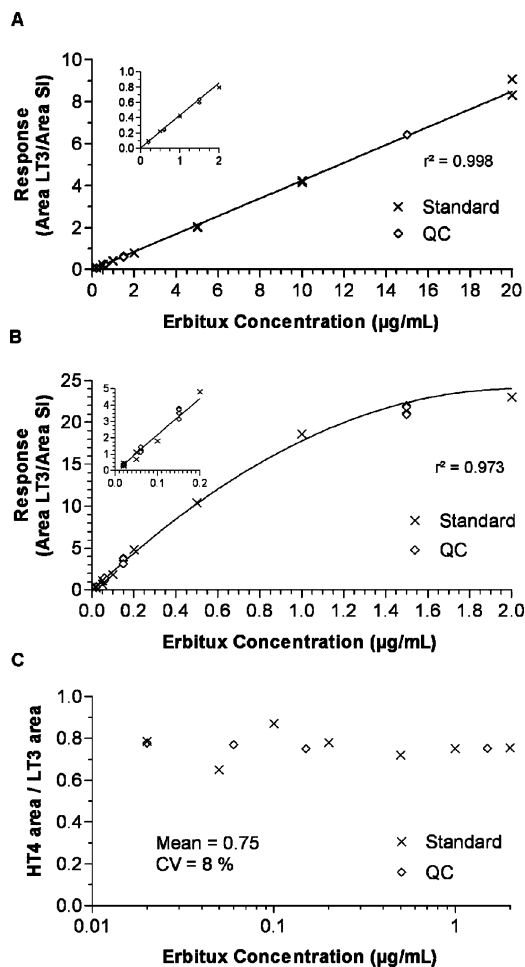


Figure 6. Calibration curves of Erbitux done by establishing a regression of the LT3 over IS peptide peak area ratio versus its concentration (A) in 50 mM Tris-HCl buffer (1 mM CaCl₂ with 100 µg/mL BSA) without immunocapture from 0.2 to 20 µg/mL, (B) in human serum with immunocapture from 20 to 2000 ng/mL. (C) HT4 over LT3 area ratio calculated after immunocapture of Erbitux samples from 20 to 2000 ng/mL.

inter-assay CVs were 17% and 12%, respectively; whereas the intra- and inter-assay accuracy was 97%, and signal-to-noise ratio of LT3 transition was 10. In the absence of the internal standard, intra- and inter-assay performance data do not significantly change except for the highest QC at 1500 ng/mL, for which significant losses of precision and accuracy could be observed with CV% and accuracy dramatically decreasing from 9% to 17% and from 86% to 75%, respectively. Results are summarized in Table 3 and show the applicability of the method to quantification of Erbitux in human serum.

Additionally, the stability of LT3, HT4, and HT23 peptides in the injection buffer was checked in the autosampler for up to 24 h. Peak area ratio was between 90% and 97%. This result showed that all three peptides were stable during the analysis.

CONCLUSION

This work presents for the first time an alternative to ELISA methods for quantification of a monoclonal antibody in biological fluids during clinical development. The approach is based on immunocapture and mass spectrometry. The initial step of the analytical process resembles that used for ELISA, where the interactions between the adsorbed target on the plate and the mAb

Table 3. Assay Performance Data of Spiked QC Samples in Human Serum at Four Levels after Immunocapture, Digestion and Measurement of LT3 over IS Area Ratio

	intra-run (<i>n</i> = 4)		inter-run (<i>n</i> = 12 over 3 days) ^a	
	CV (%)	accuracy (%)	CV (%)	accuracy (%)
LLOQ (20 ng/mL)	17	97	12	97
low QC (60 ng/mL)	17	101	18	91
mid QC (400 ng/mL)	6	105	15	93
high QC (1500 ng/mL)	9	85	9	86

^a Except mid QC (*n* = 8 over 2 days).

allow its extraction from complex biological matrix as serum. This immunorecognition of the target used in our immuno-LC-MS/MS approach is crucial, since it allows assessment of the biological potency of the antibodies. Added value is derived from mass spectrometry quantification of peptides from the variable regions and involved in the binding to sEGFR, which is highly specific since nonspecifically bound endogenous antibodies do not hamper quantification. The simultaneous monitoring of an Fc region peptide (HT23) shows the importance of this interfering phenomenon in human serum. The possibility of monitoring the whole analytical process with an appropriate internal standard is another advantage of the present mass spectrometry-based approach. Consequently, variability of the assay was below 20%. The limit of quantification of the assay at 20 ng/mL compares favorably with ELISA sensitivity, which is in the 5–5000 ng/mL range, depending upon the sample dilution required by nonspecific binding. Although this assay may appear more time-consuming than ELISA, it could be fully automated by using an online immunocapture cartridge and trypsin chamber as already developed for other analytical applications. This will imply that the stability of the covalently coupled antigen should be assessed. Other possible methodological improvements are possible. For instance, mass spectrometric quantification could be done using the more sensitive linear ion trap LTQ coupled to an Orbitrap.³⁵ The high resolution provided by this equipment enhances sensitivity and specificity. MS/MS experiments would not be necessary and could be replaced by full MS scan experiments, allowing quantification of a huge number of tryptic peptides, ensuring mAb integrity. Further identification and quantification of structural heterogeneity within the active mAb structure, such as pyroglutamic acid formation and glycosylation, are also currently in progress.

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