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Quantitative bioanalysis by LC-MS for the development of biological drugs

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Foreword

The evolution of liquid chromatography-mass spectrometry (LC-MS) for the quantification of proteins has been an extraordinary development in the field of bioanalysis. Traditionally, ligand binding assays (LBA) have been the principal quantification technique, to which LC-MS acted as a supplementary or auxiliary approach due to its limited detection sensitivity. However, LC-MS is now regarded as an established bioanalytical technique, complementary to LBA for protein quantification.

Ten years ago, LC-MS for protein quantification was in its experimental stage; today, we are witnessing its routine utilization in the majority of bioanalytical laboratories, supported by specialized, automated technologies. More recently, LC-MS has been used to support the development of biological drugs (biopharmaceuticals) via macromolecular drug and biomarker quantification, using a variety of analytical approaches such as digestion, immunocapture, chromatography and mass spectrometry. LC-MS is also becoming an important tool for anti-drug antibody (ADA), which also traditionally utilized LBA, and enzyme activity assessments.

In this eBook, we explore the current landscape of LC-MS for biopharmaceutical development, the contributions of LC-MS technologies to the wider field and the bottlenecks currently limiting further LC-MS advancement. We will also discuss the importance of accurate interpretation of concentration results, particularly for macromolecular analytes, which strongly depends on the analytical approach used.

We hope you enjoy this eBook!

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Quantitative bioanalysis by LC-MS for the development of biological drugs: an interview with Nico van de Merbel

Nico van de Merbel is Senior Director of Bioanalytical Science at ICON's Bioanalytical Laboratories in Europe and the USA. His field of expertise is the bioanalytical application of chromatographic methods for small and large molecules. He is responsible for research, method development and validation of LC-MS methods for biological samples. Nico obtained his M.Sc. in toxicology and his Ph.D. in analytical chemistry at the Free University in Amsterdam.

He holds an honorary professorship in Bioanalysis at the University of Groningen (Groningen, Netherlands). He has over 30 years of experience in quantitative bioanalysis in academia and industry and has been employed with Pharma Bio-Research/PRA/ICON since 1995.

How does quantitative LC-MS currently contribute to the field of biopharmaceutical development?

For quantitative bioanalysis of proteins, LC-MS–based approaches were first introduced a little over ten years ago. In our laboratory, and I think worldwide, the technique was initially used as a backup strategy. It was only considered if no ligand-binding assay (LBA) could be successfully developed, for example, if reagents were of insufficient quality or in cases of severe matrix effects. After a decade of developments, that situation has changed quite a bit. Although LBAs are still used for the vast majority of protein quantification methods, LC-MS is increasingly selected as a ‘first choice’ technology for all kinds of applications and reasons.

In most cases, the biopharmaceutical drug of interest is enzymatically digested and a protein-specific signature peptide is subsequently quantified as a measure for the original, intact molecule. This digestion steps breaks all existing protein-protein interactions in the sample, so LC-MS is very well suited for establishing the total concentration of a protein analyte, regardless of whether it was bound to another protein in the sample or not. Alternatively, an immunocapture step can be applied prior to LC-MS analysis, which is comparable with the first binding step of an LBA. With such a hybrid approach, it may be possible to determine the free fraction of a protein, or at least the fraction that is captured by the binding reagent. In addition, LC-MS is often used in situations where the development of critical immunological reagents is undesirable because it is considered too time-consuming or too costly, for example in early preclinical development. This is especially interesting for monoclonal antibody drugs, because a peptide can be selected for quantification, which occurs in all drug candidates of a specific structural subclass, but which is not present in the endogenous proteins in the animal species.

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In this way, a single generic LC-MS method can be used for quantifying multiple biopharmaceuticals in a variety of animal samples. Altogether, LC-MS has certainly found its place in the field of biopharmaceutical development, not only for quantifying the drug itself, but also increasingly for measurement of large molecule biomarkers.

What sets LC-MS apart from other methods for quantitative bioanalysis in drug development?

I believe the value of LC-MS for biopharmaceuticals and other proteins lies in the fact that it provides information that is complementary to the results of an LBA . While an LBA response is based on the binding characteristics of a protein, LC-MS is directly related to its molecular structure and it is the combination of results that can improve our understanding of the in vivo fate of these molecules. In particular, LC-MS is helpful when detailed information is required about the structural aspects of a protein. For example, we are seeing increased interest in monitoring the concentrations of specific protein forms that an LBA may not be able to distinguish from the main form. In vivo biotransformation reactions can lead to small structural changes, by truncation, deamidation or otherwise, that may impact the pharmacological activity of a biopharmaceutical drug. By selecting the appropriate signature peptide, LC-MS is often capable of distinguishing these closely related protein forms. In the most extreme case, forms that only differ in their 3D orientation can be determined separately.

The same is true for the isoforms of biomarkers. Naturally occurring mutants, which are just one amino acid different, can be measured relatively easily next to the wildtype form. This brings me to a very clear advantage of LC-MS: its ability for multiplexed analysis. In principle, there is no limit to the number of peptides that can be followed in a single analysis; it is quite straightforward to quantify multiple proteins together, each by reference to its own signature peptide. Depending on the situation, these can be several drugs, several biomarkers or a combination. In addition, an LC-MS assay can include more than one signature peptide per protein. This is useful for studying the biotransformation of a biopharmaceutical drug, by following both the unchanged and the changed part of the molecule. Alternatively, the objective can simply be to increase the percentage of the molecular structure that is covered by the signature peptides. From a technical point of view, LC-MS also has other advantages that can be helpful for routine analysis, such as the generally wider linear dynamic range than an average LBA, which reduces the need for multiple dilution factors. The opportunity to use internal standards for response normalization also often increases method performance.

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What role will LC-MS technology ultimately play in this field and can you foresee any new fields of application for the future?

For the foreseeable future, it is likely that LBA will remain the bioanalytical workhorse for protein quantification, especially for straightforward applications, due to their superior sensitivity, higher sample throughput and lower operational costs. However, we are seeing a clear trend indicating LC-MS is preferential for answering more complicated questions, especially those related to the molecular structure of a protein. For example, we are beginning to see an interest in the glycosylation patterns of both biopharmaceuticals and endogenous proteins in biological samples and, in some cases, it can be important to confirm the presence of a specific structural element within the protein, such as the phosphorylation of a certain amino acid.

Secondly, LC-MS will remain a useful option when it is difficult to generate reagents for an LBA with sufficient specificity. An interesting further development is the use of LC-MS as an alternative readout for an LBA. One example is the assessment of anti-drug antibodies, which are present in a sample in case the body develops an immune response towards a biopharmaceutical drug. The first step in such an approach is the binding of these antibodies by the immobilized drug, just like in a traditional LBA. After this capturing step, the multiplexing capabilities of LC-MS allow the simultaneous measurement of the different anti-drug antibody subclasses, each by a separate signature peptide, and this provides more detailed information about anti-drug antibody formation.

One final, relatively new approach I'd like to mention is the use of LC-MS for the quantification of intact proteins. Even though digestion of a protein and subsequent analysis of one or more signature peptides has shown its merit for protein quantification, in many cases the signature peptides represent a small fraction of the protein, sometimes no more than a few percent, and information about the remaining part of the molecule is therefore lost. However, LCMS is also capable of measuring intact proteins, typically using high-resolution mass spectrometry. The combination of an immunocapture step and this type of LC-MS allows quantitation of proteins as large as monoclonal antibodies. In this way, information can be obtained that is complementary to both LBA and to digested protein analysis by LC-MS.

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What do you consider to be the technical bottlenecks that still need to be addressed to move LC-MS technology forward?

After a decade of using LC-MS for protein quantification, we realize that its main drawback compared with LBA is the limited detection sensitivity. Enzymatic digestion followed by direct analysis of the digest typically allows quantification in the µg/mL range. After sample cleanup by an immunocapture step, concentrations down to low ng/mL or perhaps high pg/mL can be covered, but analysis at lower levels remains a challenge. In addition, the efficiency of the chromatographic separation of intact proteins is less favorable than what we are used to for small molecules and peptides, so there is room for improvement there as well. It would also be very useful if the overall throughput of an LC-MS method could be increased. Many methods currently use lengthy steps for immunocapture and digestion, which may lead to a two-day assay or at least to a very long one-day assay. Finally, although it is not a technical bottleneck, there still is not a lot of clarity regarding what regulatory authorities expect in terms of the performance of bioanalytical LC-MS methods for proteins, when these are used to support drug development. Those of us working in the field of regulated bioanalysis would therefore also benefit from more regulatory guidance.

What have been the major advancements to LC-MS techniques over the last 5 years?

The most important advancement is perhaps not technical, but rather conceptual. I believe that protein LC-MS has matured in the sense that we now often have a better understanding of the actual meaning of a concentration result. Through working jointly with proteins scientists, it has become increasingly clear that proteins are by no means homogeneous and inert molecules. From a chemical and 3D point of view, a protein is often a series of closely related molecules with all kinds of small structural differences, each of which may change during residence in the body and may have a different pharmacological activity or, for a biomarker, biological significance. Different bioanalytical techniques are typically targeted to different structural elements of that family of molecules. This means that it is not unusual to find different concentration results for the same sample when various techniques are applied. Not only are differences common when comparing an LBA with LC-MS, but two LC-MS methods or two LBAs with different principles may very well have dissimilar outcomes.

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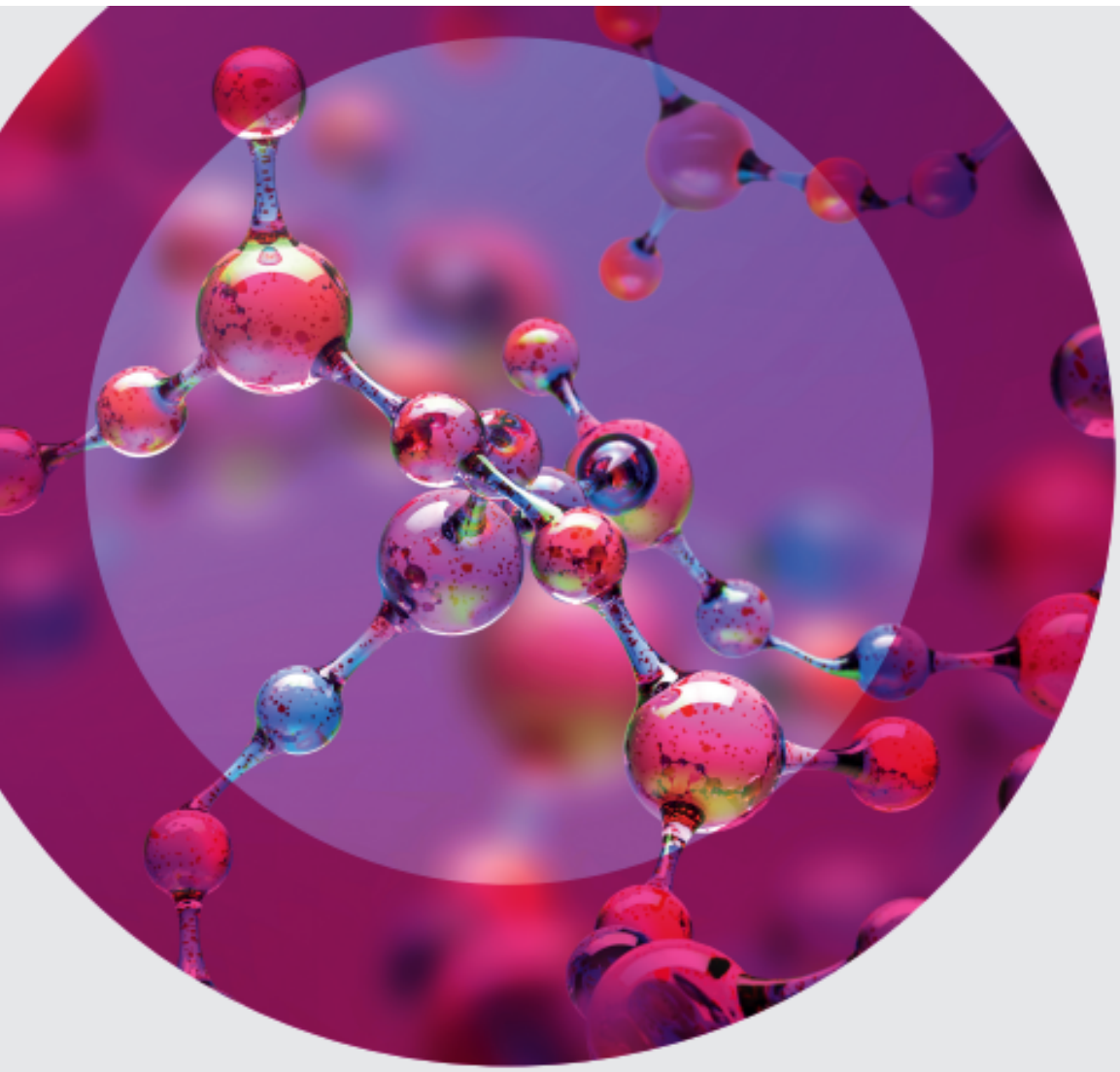
It is important to realize that these differences are not necessarily an indication that one or more of the methods provide incorrect results. On the contrary, proteins are often so large and complicated that they do not have a single, well-defined concentration and it takes multiple analytical approaches, directed to the different relevant parts of their structure, to obtain a complete picture of their bioanalytical behavior. Before developing a method, it should therefore be clear what kind of kind of concentration result offers most value in a particular research context, so that the proper analytical approach, or approaches, can be selected. I believe that, compared to 5 years ago, this is now more common practice.

How did you become involved in the field and why would you encourage early career scientists to specialize in LC-MS?

I know it is a cliché, but I firmly believe that it is always best to work in an area that one really enjoys. What I like about LCMS is that it is an information-rich technology. It allows you to approach a complicated situation and answer difficult questions in a structured and analytical way. If that is what you enjoy too, LCMS is certainly worth considering for your own career.

I was trained as an analytical chemist and I have been working in the field of quantitative bioanalysis for over 30 years, starting my career before LC-MS became a routine technique. I had the opportunity to help introduce the technology into our laboratory, first for small molecules and in the past decade also for proteins and other macromolecules. To support this, ICON entered into a long-term collaboration with the analytical biochemistry group of the nearby University of Groningen in the Netherlands and I believe this partnership has been really beneficial for both parties. Together, we had the opportunity to develop our technical skills as well as our theoretical knowledge and, by applying both to projects from our customers, I hope it has contributed to the interesting field of biopharmaceutical drug development.



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Research Article

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Bioanalysis

Improving selectivity and sensitivity of protein quantitation by LC–HR–MS/MS: determination of somatropin in rat plasma

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Aim: Protein quantitation by digestion of a biological sample followed by LC–MS analysis of a signature peptide can be a challenge because of the high complexity of the digested matrix. **Results/methodology:** The use of LC with high-resolution (quadrupole-TOF) MS detection allowed quantitation of the 22-kDa biopharmaceutical somatropin in 60 µl of rat plasma down to 25 ng/ml with minimal further sample treatment. Reducing the mass extraction window to 0.01 Da considerably decreased the interference of tryptic peptides, enhanced sensitivity and improved accuracy and precision. Analysis with LC–MS/MS resulted in a less favorable limit of quantitation of 100 ng/ml. **Conclusion:** HRMS is an interesting option for the quantitation of proteins after digestion and has the potential to improve sensitivity with minimal method development.

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Keywords: digestion • high-resolution mass spectrometry • HRMS • mass extraction window • MEW • rhGH • somatropin

LC in combination with MS/MS, performed on a triple quadrupole mass spectrometer in the multiple reaction monitoring mode, has been the gold standard for over two decades when it comes to the quantification of small molecules and peptides in biological matrices. This technique is known for its robustness, high selectivity, sensitivity and reproducibility, and is used as the bioanalytical workhorse in laboratories across the globe.

An important new development of the past few years is the application of LC–MS/MS for the quantification of proteins, as an alternative for, or an addition to, the traditional ligand-binding assays [1–3]. Although LC–MS/MS for proteins has many technical advantages, such as improved precision and accuracy, a larger linear dynamic range, and independence of critical immunological reagents which may suffer from cross-reactivity and batch-to-batch differences, it is typically inferior to ligand-binding assays in terms of sensitivity [4]. One of the reasons for this is related to the fact that large proteins need to be digested to peptides of much reduced size to be compatible with the mass range of a triple quadrupole mass spectrometer. If a protein-rich matrix such as serum or plasma is digested, a highly complex sample is obtained containing a multitude of peptides, which are all composed of the same limited number of natural amino acids and, thus, have masses and properties that are much more similar to one another than those of the original proteins. As a result, many peaks often show up in the multiple reaction monitoring chromatograms, which greatly impacts method selectivity and typically limits the obtainable sensitivity for protein analytes to the low µg/ml or high ng/ml range [5–7]. In a recent paper, the magnitude of this effect was studied for salmon calcitonin [8]. The lower limit of quantitation (LLOQ) for digested plasma was found to be a factor of 100 higher than that for a digested test solution and this was only due to the presence of interfering matrix peaks in the LC–MS/MS chromatograms. For obtaining better sensitivity, the usual approach is to remove interfering peptides by applying extraction procedures at the protein and/or peptide level, such as immunodepletion, immunocapture and solid-phase extraction [9–11]. Although this can improve sensitivity considerably, even down to the pg/ml

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range [12,13], the required research will add to method development time and the additional steps may negatively impact accuracy and precision.

The limited selectivity of MS/MS detection for digested biological samples is related to the relatively low mass resolution of a quadrupole mass spectrometer and the corresponding rather wide mass extraction window (MEW) that is used to select analyte ions for detection. The typical setting in MS/MS is unit–mass resolution for both quadrupoles with a MEW corresponding to full width at half maximum of 0.7 Da, which allows distinction between ions with m/z values that are one unit apart. While this generally provides appropriate selectivity for small-molecule analytes, the strong similarity of the peptides in biological sample digests, the frequent occurrence of multiple charges and their comparable fragmentation patterns, makes it highly likely that multiple precursor and product ions will be generated with very close m/z values, which all will be selected in the triple quadrupole mass spectrometer and, ultimately, will be detected and give a response in the chromatograms. Although many of these peptides can be chromatographically separated from the surrogate peptide released by the analyte, interferences at the same retention time as the surrogate peptide will often be unavoidable, and this will negatively impact the selectivity and sensitivity of the method.

An obvious approach to reduce interferences is to select a narrower MEW for detection, a situation which can be provided by HRMS. Typically, HRMS is run in full-scan mode, which records all sample ions. Extracted-ion chromatograms are subsequently constructed by setting an MEW around the theoretical m/z of the analyte ion and recording the response of all ions within the MEW. In contrast to detection on a triple quadrupole, in HRMS the magnitude of the MEW can be easily reduced, and the sensitivity and selectivity of the detection optimized. In recent years, the field of HRMS has seen considerable improvements with regard to sensitivity, linear dynamic range and scan modes, and the technique is increasingly seen as a serious option for quantitative bioanalysis, even though the instrument sensitivity of triple quadrupole MS typically still is superior [14,15]. So far, most published applications of HRMS in quantitative bioanalysis have been for small molecules or peptides [16–19] and although the applicability of HRMS has also been demonstrated for protein quantification after digestion [20–25], this field clearly still is in its infancy and a systematic investigation of the potential of HRMS to improve selectivity is still lacking.

In this paper, we present and evaluate a quantitative LC–HR-MS/MS method for the 22-kDa biopharmaceutical protein somatropin (recombinant human growth hormone) in rat plasma. After trypsin digestion, three signature peptides from different parts of the protein molecule are quantified on LC coupled to a quadrupole-TOF (Q-TOF) mass spectrometer, which combines unit–mass resolution for selection of the precursor ion (quadrupole) and high resolution for selection of the product ion after fragmentation (TOF). The effect of the MEW and of the summing up of multiple ions on assay performance (precision, accuracy, selectivity and sensitivity) of all three peptides is described and a comparison to the performance of a triple quadrupole MS is provided. The applicability of the optimized HR–MS/MS approach is illustrated by analysis of rat plasma samples from a pharmacokinetic study.

Experimental section

Chemicals & materials

Recombinant human growth hormone (somatropin), supplied as a lyophilized sterile powder, was obtained from Ferring (Copenhagen, Denmark). Information on the amino acid sequence is given in Figure 1 . Custom synthesized internal standard peptides (SNLELLR and LFDNAMLR with a $^{13}C_6$-$^{15}N_4$-labeled C-terminal arginine and FDTNSHNDDALLK with a $^{13}C_6$-$^{15}N_2$-labeled C-terminal lysine) were obtained from JPT Peptide Technologies (Berlin, Germany). Acetonitrile, methanol, 2-propanol, formic acid, acetic acid, ammonia (25%) and hydrochloric acid (37%) were obtained from Merck (Darmstadt, Germany). Tween-20, Trizma® base and trypsin from porcine pancreas (Type IX-S, lyophilized powder, 13,000–20,000 BAEE units/mg protein) were obtained from Sigma-Aldrich (MO, USA). HPLC grade water was prepared using a water purification system from Merck. Rat EDTA plasma (hereafter referred to as blank rat plasma) was obtained from Seralabs (Haywards Heath, UK). ESI Positive Calibration Solution for the Q-TOF system was obtained from Sciex (Toronto, Canada).

Preparation of calibration & quality control samples

A somatropin stock solution at 10.0 mg/ml was prepared by dissolving the contents of a vial of lyophilized protein (label claim: 10.0 mg) in 1.00 ml of water according to the manufacturer's instructions for use. The stock solution was divided into 0.2 ml aliquots in Eppendorf Protein Lo-bind tubes (obtained from VWR International, Amsterdam, The Netherlands) and stored at -80°C. Two sets of aqueous standard solutions at 2.50, 25.0 and

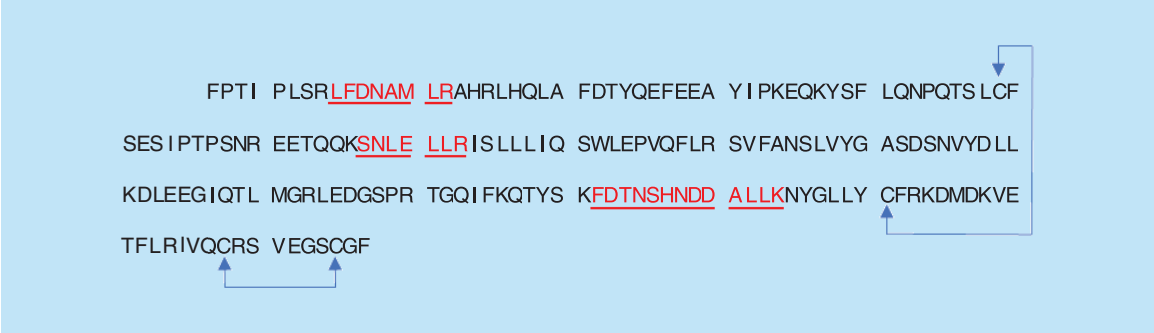

Figure 1. Amino acid sequence of somatropin.
The three signature peptides are highlighted, and the disulfide bonds shown as arrows.

200 µg/ml were prepared freshly from this stock solution. One set was used to prepare calibration samples in blank rat plasma at 25.0, 50.0, 100, 125, 250, 500, 1250, 2000, 5000, 8000 and 10,000 ng/ml. Similarly, quality control (QC) samples were prepared from the other set at 25.0, 50.0, 75.0, 1250 and 8000 ng/ml. All calibration and QC samples were stored in Eppendorf Protein Lo-bind tubes at -80°C.

Sample pretreatment

Aliquots of 60 µl of rat plasma were pipetted into the 1.2-ml wells of an Eppendorf Protein Lo-bind 96-well plate (VWR International) and 200 µl of an 80:20 (v/v) mixture of acetonitrile and water was added. After vortex-mixing for 2 min, the proteins were pelleted by centrifugation for 10 min at 1500 × *g*. The supernatant was discarded by inverting the plate above a waste receptacle and placing it upside down on a tissue for 15 min. The protein pellet was reconstituted by vortex-mixing in 400 µl of a digestion solution consisting of 50 mM Trizma buffer (pH 8.0), 2.5% (v/v) acetonitrile and 200 µg/ml trypsin in water. Next, 60 µl of the digestion solution, excluding trypsin and containing 8000 ng/ml of the internal standards was added. The sample was digested at 37°C and 1250 rpm for 90 min using an Eppendorf (Hamburg, Germany) Thermomixer® comfort, after which the digestion was stopped by the addition of 25 µl of 10% formic acid in water. The complete digest was transferred onto an Oasis PRiME HLB 96-well plate with 30 mg sorbent (Waters, MA, USA). The SPE columns were washed with 800 µl methanol/water (5:95, v/v) and eluted with 250 µl methanol/water (50:50, v/v), followed by 250 µl 5% ammonia in methanol/water (50:50, v/v) into an Eppendorf Protein Lo-bind 96-well plate. The combined eluate fractions were evaporated to dryness under nitrogen at 60°C (upper) and 80°C (lower) using a Biotage (Uppsala, Sweden) SPE Dry 96 dual sample concentrator system and reconstituted in 60 µl 0.1% formic acid in acetonitrile/water (5:95, v/v). Finally, the plate was sealed, vortex-mixed and placed in an autosampler at 10°C for analysis.

Chromatography

Digested samples were analyzed using a NexeraX2 system (Shimadzu, Tokyo, Japan) coupled to a Sciex TripleTOF 6600 Q-TOF mass spectrometer equipped with a Dual Spray source or using a 1290 Infinity II system (Agilent, CA, USA) coupled to a Sciex TripleQuad 6500 triple quadrupole mass spectrometer equipped with a IonDrive TurboV source.

Chromatographic separation was performed at 60°C on a 2.1 × 100 mm (particle size 1.6 µm, pore size 100 Å) Luna Omega C18 column (Phenomenex, CA, USA). Mobile phase A consisted of 0.1% formic acid in a mixture of water and 2-propanol (95:5, v/v) and mobile phase B was 0.1% formic acid in a mixture of acetonitrile and 2-propanol (95:5, v/v). Gradient elution was performed at 0.6 ml/min using the following profile: 0.0–6.0 min: 0–13% B; 6.0–6.2 min: 13–55% B; 6.2–6.7 min: 55% B; 6.7–6.9 min: 55–95% B; 6.9–7.9 min: 95% B; 7.9–8.1 min: 95–0% B; 8.1–11.5 min: 0% B. The injection volume was 15 µl. The mobile phase was diverted to waste between 0 and 1.5 min and between 8 and 11.5 min using a VICI (TX, USA) switching valve.

Mass spectrometry

The Q-TOF mass spectrometer was operated in high sensitivity mode at a resolution of approximately 20,000 at m/z 1000 and in ESI positive ion mode. Mass spectrometric settings were optimized by infusion of the labeled peptides into the mass spectrometer. Specific settings are included in Table 1. All mass spectra were collected

Table 1. Amino acid sequence, precursor and product ion m/z, charge states and analyte specific MS-settings for the signature peptides and their stable-isotope-labeled internal standards.

Peptide sequence	Triple Quad MS/MS and HR–MS/MS precursor ion mass (m/z) and charge state	Triple quad MS/MS product ion mass (m/z) and charge state	HR–MS/MS extracted product ion mass (m/z), charge state and extraction window (Da)	CE (V)	Triple quad MS/MS dwell time (ms)	HR–MS/MS accumulation time (ms)
FDTNSHNDDALLK	497.2/$(M + 3H)^{3+}$	671.8/y_{12}^{2+}	614.3090/y_{11}^{2+}/0.01, 0.07 and 0.5 671.8236/y_{12}^{2+}/0.01, 0.07 and 0.5	22	50	40
FDTNSHNDDALLK†	500.0/$(M + 3H)^{3+}$	675.8/y_{12}^{2+}	618.3152/y_{11}^{2+}/0.01 675.8308/y_{12}^{2+}/0.01	22	50	15
SNLELLR	422.8/$(M + 2H)^{+}$	643.4/y_5^{+}	401.2871/y_3^{+}/0.01, 0.07 and 0.5 530.3328/y_4^{+}/0.01, 0.07 and 0.5 643.4200/y_5^{+}/0.01, 0.07 and 0.5	20	50	40
SNLELLR‡	427.8/$(M + 2H)^{2+}$	653.4/y_5^{+}	411.2956/y_3^{+}/0.01 540.3419/y_4^{+}/0.01 653.4290/y_5^{+}/0.01	20	50	15
LFDNAMLR	490.3/$(M + 2H)^{2+}$	719.4/y_6^{+}	604.3271/y_5^{+}/0.01, 0.07 and 0.5 719.3563/y_6^{+}/0.01, 0.07 and 0.5 866.4330/y_7^{+}/0.01, 0.07 and 0.5	25	50	40
LFDNAMLR‡	495.3/$(M + 2H)^{2+}$	729.4/y_6^{+}	614.3369/y_5^{+}/0.01 729.3678/y_6^{+}/0.01 876.4395/y_7^{+}/ 0.01	25	50	15

†C-terminal lysine $^{13}C_6{}^{15}N_2$-labeled internal standard.
‡C-terminal arginine $^{13}C_6{}^{15}N_4$-labeled internal standard.

in profile mode with the start and end mass set respectively at 350 and 900 m/z. Analyst TF software 1.7.1 in combination with MultiQuant 2.0.1 and PeakView 3.0.2 (Sciex) were used for data acquisition and processing. The system was calibrated by application of the standard calibration mixture through the calibrant delivery system unit prior to every batch and during analysis after every 6 injections.

The triple quadrupole mass spectrometer was operated at unit–mass resolution in ESI positive ion mode. Analyst software 1.6.2 was used for data acquisition and processing. For both MS systems the following settings were used: IS voltage was set at 5500 V, source temperature at 700°C and the DP at 60 V. Curtain gas value was 35 psi. For the nebulizer and drying gas 50 and 50 psi were used, respectively. Collision-activated dissociation gas was set at 9.

Pharmacokinetic study

Sprague Dawley rats were dosed under appropriate ethical approval with a single 2 mg/kg subcutaneous bolus injection of somatropin (Zomacton®). Blood samples were collected in K_3-EDTA tubes prior to dosing and at 0.5, 1, 2, 4, 8 and 24 h post dose. Plasma was prepared immediately after blood collection and stored at -80°C until analysis.

Results & discussion

Selection of signature peptides & method optimization

An *in silico* tryptic digestion of the somatropin sequence with Skyline [26] was used to generate a list of, in total, 17 candidate signature peptides. To avoid interferences from the rat plasma proteome, all signature peptides had to be unique for somatropin, which was checked by submitting the preliminary peptide list to the Basic Local Alignment Search Tool (BLAST) version 2.2.29 [27]. In addition, to facilitate LC–MS quantitation and avoid reduction and alkylation steps, peptides forming disulfide bonds were excluded as well as peptides outside the 300–2000 mass range.

For an extensive evaluation of the applicability of HRMS for the analysis of plasma digests, we wanted to include multiple peptides. Since the three peptides LFDNAMLR (amino acids 9–16), SNLELLR (71–77) and FDTNSHNDDALLK (146–158) fulfilled all criteria, these were evaluated for further use, also because they represent different parts of the protein structure (C-terminal region, center and N-terminal region, respectively) and because the first peptide contains an oxidizable methionine [28]. Together they will therefore provide extended information about the *in vivo* fate of the protein drug. To confirm the suitability for LC–MS analysis, peptide mapping experiments were conducted using a tryptic digest of a test solution of somatropin after protein precipitation with acetonitrile. Since all three peptides showed appropriate LC–MS characteristics such as good chromatographic retention, efficient

ionization and fragmentation and peptide LFDNAMLR also showed negligible *in vitro* oxidation, they were selected for further work. A multistep chromatographic gradient was optimized to achieve good peak shape, retention and separation of the peptides (retention times 2.0, 4.2 and 5.6 min for peptides LFDNAMLR, SNLELLR and FDTNSHNDDALLK, respectively). Analyte-specific mass spectrometric parameters are presented in Table 1, as well as the mass transitions used to monitor the peptides and the corresponding stable-isotope labeled internal standards.

Sample preparation was kept as basic as possible to maintain digest complexity and allow optimal evaluation of the effect of the mass spectrometric settings on method selectivity. After protein precipitation of spiked plasma with acetonitrile, pellet digestion performed at pH 8.0 and 37°C and in the presence of 2.5% acetonitrile was complete after 90 min when using 200 µg/ml of trypsin. To increase sensitivity, the relatively large digest volume of 425 µl was reduced to 60 µl by using reversed-phase SPE, evaporation and reconstitution. The recovery of all signature peptides was about 75%. Although very polar and very nonpolar matrix peptides may not have been recovered from the digest because of their breakthrough and nonelution, respectively, the generic SPE approach is expected not to introduce a high degree of selectivity. In particular, matrix peptides eluting at or around the retention times of the signature peptides on the reversed-phase LC column, are not likely to have been removed from the digest using the described procedure.

Reduction of MEW & summation of product ions

The effect of the MEW value on method selectivity is exemplified in Figure 2A for peptide SNLELLR, after digestion of plasma spiked at the desired LLOQ of 25 ng/ml of somatotropin. The doubly charged precursor ion (m/z 422.8) was selected in the quadrupole and extracted ion chromatograms were constructed around product ion m/z 401.2871, which corresponds to the singly charged y_3 ion. At an MEW of 0.5 Da, which simulates the typical settings of a triple quadrupole, the signature peptide peak is obscured by a wide range of interfering peaks (Figure 2A-1). This illustrates the enormous complexity of the plasma digest and the high similarity of the signature peptide to tryptic peptides that are generated from the plasma proteome. Reducing the MEW to 0.07 Da results in a slight decrease of the signature peptide peak intensity, but more importantly causes a much more pronounced reduction of the interferences (Figure 2A-2). By further narrowing the MEW to 0.01 Da, an additional decrease of the interfering peaks is obtained and although the signature peptide response is also further reduced, the resulting signal to noise ratio is more favorable than for the wider MEWs (Figure 2A-3).

The cause of this phenomenon is illustrated in Figure 2B. The quadrupole, which operates at unit–mass resolution and a MEW of 0.7 Da, selects precursor ions in the m/z range of 422.8 ± 0.35. After fragmentation of these precursor ions, the TOF mass analyzer, when used at a MEW of 0.5 Da (Figure 2B-1), captures all y_3 ions of the signature peptide, but at the same time also all other matrix product ions in the m/z range of 401.2871 ± 0.25. At the retention time of the signature peptide alone, at least two other (mass-resolved) peptides contribute to the detection signal and across the entire chromatogram this amounts to more than 30 peptides. The mass resolution of the TOF that was used in this work is approximately 20,000 and at this value a MEW of 0.07 Da extracts the majority of the signature peptide y_3 ions. Within the selected m/z range of 401.2871 ± 0.035, it appears that one other peptide product ion is partially co-extracted (Figure 2B-2). Optimal selectivity is obtained at a MEW of 0.01 Da, or a m/z range of 401.2871 ± 0.005 (Figure 2B-3). The mass spectrum corresponding to the retention time of the signature peptide indicates that no other matrix peptides are extracted within this window. Therefore, optimal accuracy is obtained with this MEW, whereas at larger MEW values, an overestimation of the signature peptide concentration occurs. A drawback of this setting is that only a relatively small part of formed y_3 ions is selected for detection and that the absolute sensitivity, therefore, is also reduced. For the other product ions for peptide SNLELLR, as well as for the product ions of the other two peptides LFDNAMLR and FDTNSHNDDALLK, comparable results were obtained and narrowing the MEW led to improved selectivity, with cleanest chromatograms generally being found for an MEW of 0.01 Da.

An interesting feature of HRMS is the fact that the responses that are obtained for different analyte-related ions can easily be summated post acquisition to enhance detection sensitivity. Figure 3 illustrates the advantages and disadvantages of this approach. For peptide SNLELLR, three product ions (y_3, y_4 and y_5) are generated with useful intensities after collision-induced dissociation of the precursor ion, and combining the signals of these product ions leads to an increased analyte response. The summation of y_3 and y_4 causes an approximately 50% increase of the peak area compared with capturing only y_3 (Figure 3A & B), while after the further inclusion of y_5 the final peak area is about twofold higher than for y_3 alone (Figure 3C). However, the summation of ion intensities

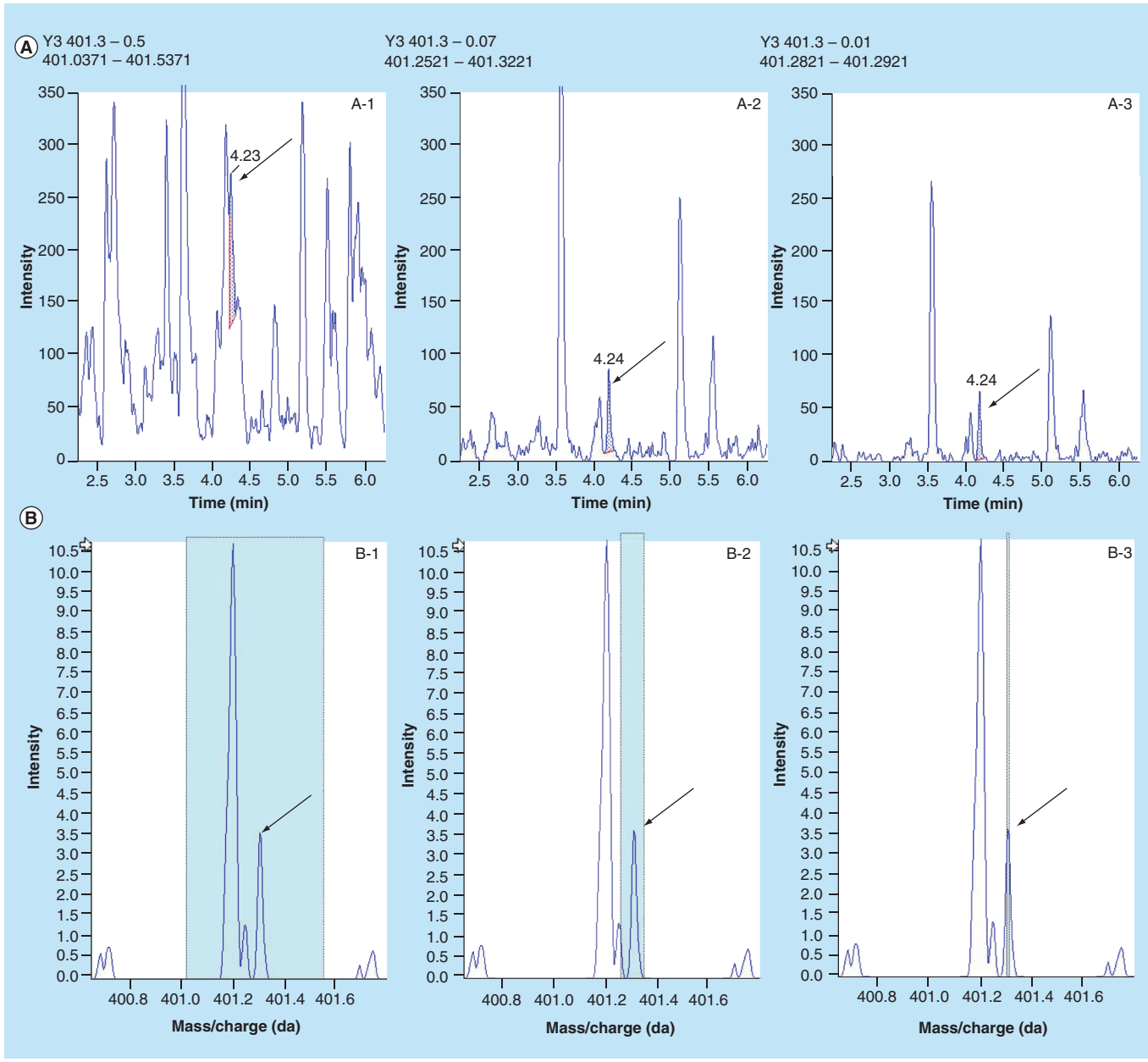

Figure 2. Extracted ion chromatograms for signature peptide SNLELLR after digestion of rat plasma spiked with somatropin at 25 ng/ml (A) and corresponding product ion mass spectra (m/z 401.2871 (y_3^+) at the retention time of the analyte (B); mass extraction window of 0.5 Da (A-1, B-1), 0.07 Da (A-2, B-2) and 0.01 Da (A-3, B-3). Arrows indicate retention time **(A)** or m/z **(B)** of the analyte. Precursor ion: m/z 422.8.

not only causes an increase in analyte peak intensity, but also of the abundance of matrix interferences. Clearly, addition of the response corresponding to the y_4 ion (m/z range: 530.3328 ± 0.005) and of the y_5 ion (m/z range: 643.4200 ± 0.005) introduces a number of additional peaks in the extracted ion chromatograms. Altogether, this demonstrates that summation of the responses of different ions may help improve sensitivity but, depending on the actual m/z values and extraction windows used, the signal-to-noise ratio may not increase or may even decrease. In addition, accuracy and precision may or may not be affected. Consequently, whether summation of ion intensities is beneficial needs to be determined case by case.

Here, two important other remarks have to be made. First, the intensity of the ions to be summated should be approximately equal or at least not be too different. At low analyte levels, ions with insufficient intensity may

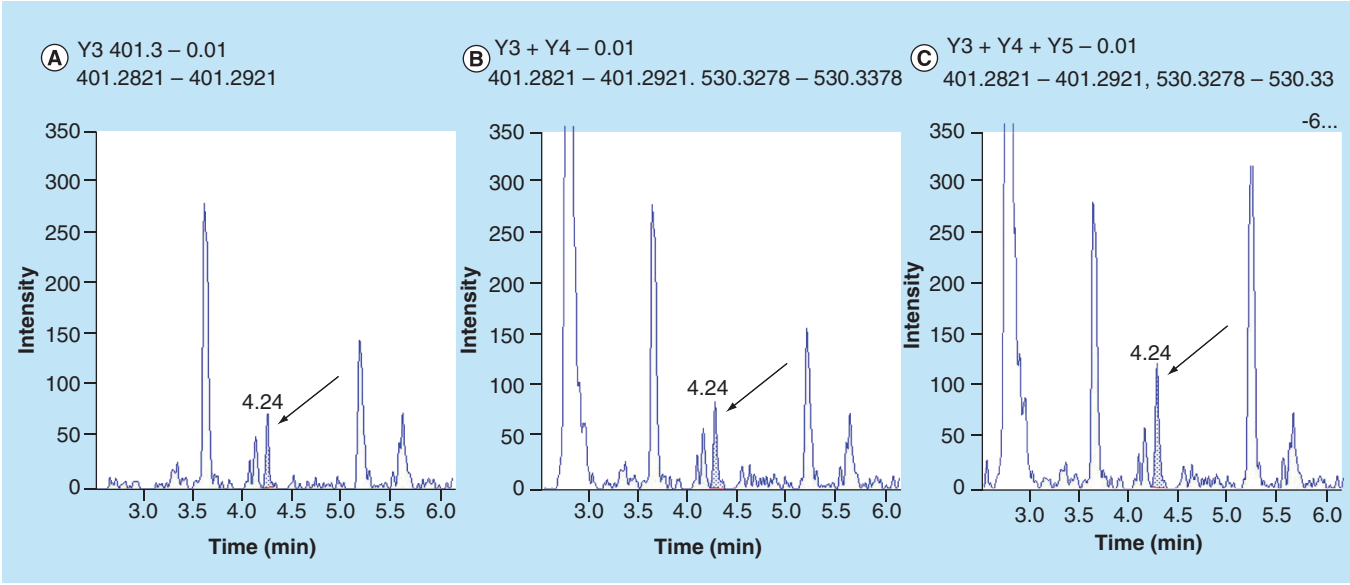

Figure 3. Extracted ion chromatograms (mass extraction window 0.01 Da) for signature peptide SNLELLR after digestion of rat plasma spiked with somatropin at 25 ng/ml; precursor ion: m/z 422.8, product ions: y_3: m/z 401.2871, y_4: m/z 530.3328, y_5: m/z 643.4200. (A) response for y_3 only, **(B)** response for the sum of y_3 + y_4, **(C)** response for the sum of y_3 + y_4 + y_5. Arrows indicate retention time of the analyte.

otherwise be lost, not included in the summation and results may thus be inconsistent with those at higher levels. In the current work, this was no issue for any of the peptides of somatropin. Furthermore, if different charge states of an ionized analyte are to be summated, it should be realized that the charge state distribution of a peptide or protein can change due to differences in experimental conditions (such as pH or ionic strength of the mobile phase, MS source settings or analyte concentration) [29]. Although for tryptic peptides not many charge states of equal sensitivity typically occur, this effect may also complicate straightforward analyte quantification and should be carefully investigated. For the three tryptic peptides of somatropin only one charge state was encountered for the precursor ion, so this effect did not play a role here.

Accuracy & precision

The gain in selectivity at lower values of the MEW is directly related to method performance in terms of accuracy and precision. Table 2 shows the results for the different product ions of peptide SNLELLR at different MEW values, for samples spiked with somatropin at the desired LLOQ of 25 ng/ml. For y_3, y_4 as well as y_5, the presence of the interferences at an MEW of 0.5 Da generally resulted in unfavorable values for accuracy (up to 630%) and precision (CV up to 60%). The responses of the two co-eluting peptides (see Figure 2) led to a considerable overestimation of the added analyte concentration, while the high variability in the results was a result of the difficulty in consistently integrating the analyte peak in the presence of an elevated background.

Narrowing the MEW to 0.07 Da and further to 0.01 Da improved the accuracy and precision found for nearly all y-ions or combinations of ions to values that meet international acceptance criteria for small molecules of ±20% at the LLOQ level. Clearly, the decrease of the interfering signals caused by these narrower MEW's resulted in a strongly reduced overestimation of analyte levels and a more reproducible peak integration. For peptide SNLELLR, we selected the sum of the responses for the y_3 and y_4 product ions at a MEW of 0.01 Da as the final approach for quantitation. Although other combinations also resulted in acceptable accuracy and precision, the chromatograms corresponding to this setting allowed the most straightforward integration and this was deemed the most robust compromise between the required selectivity and sensitivity.

For peptides FDTNSHNDDALLK and LFDNAMLR a comparable situation was found, as shown in the Supplementary Data (Tables 1 & 2). At a MEW of 0.5 Da, the signature peptide peak in some cases disappeared into the background signal, leading to underestimation of analyte levels (accuracies <75%). The detection sensitivity found for FDTNSHNDDALLK was lower than for the other two peptides and reducing the MEW to 0.01 Da

Table 2. Summary of precision (CV) and accuracy results for peptide SNLELLR under different HR-MS/MS settings (mass extraction window); somatropin concentration in plasma: 25 ng/ml, n = 6.

Product ion	Extraction window (Da)	CV (%)	Accuracy (%)
y_3 (m/z 401.2871)	0.5	61.4	222.0
	0.07	6.0	104.2
	0.01	19.6	94.4
y_4 (m/z 530.3328)	0.5	39.4	106.1
	0.07	18.8	108.1
	0.01	21.8	109.3
y_5 (m/z 643.4200)	0.5	31.0	631.1
	0.07	25.0	135.9
	0.01	19.3	88.5
$y_3 + y_4$	0.5	21.7	209.2
	0.07	9.5	97.6
	0.01	11.9	103.3
$y_3 + y_5$	0.5	19.3	420.8
	0.07	23.1	89.3
	0.01	24.4	84.0
$y_4 + y_5$	0.5	37.8	318.9
	0.07	10.4	97.6
	0.01	7.3	92.7
$y_3 + y_4 + y_5$	0.5	33.2	467.6
	0.07	12.7	91.5
	0.01	8.5	82.6

Table 3. LLOQ, summary of precision (CV) and accuracy results for the final HR-MS/MS settings for all three peptides; n = 6.

Peptide	Precursor ion (m/z)	Product ions (m/z)	MEW (Da)	Concentration (ng/ml)	CV (%)	Accuracy (%)
FDTNSHNDDALLK	497.2	614.3090 (y_{11}) + 671.8236 (y_{12})	0.07	25 50 75 1250 8000	15.7 10.6 4.4 5.4 6.4	106.6 103.7 110.1 95.1 88.9
SNLELLR	422.8	401.2871 (y_3) + 530.3328 (y_4)	0.01	25 50 75 1250 8000	11.9 10.4 10.9 9.0 6.8	103.3 98.7 99.6 97.3 91.7
LFDNAMLR	490.3	604.3271 (y_5) + 719.3563 (y_6) + 866.4330 (y_7)	0.01	25 50 75 1250 8000	11.3 16.3 7.2 12.7 1.8	88.6 80.8 110.2 105.4 108.3

resulted in peaks that were too small to be accurately quantified. Instead, an MEW of 0.07 Da was used as a compromise between sensitivity and selectivity. For this peptide, the combination of the two product ions y_{11} and y_{12} gave the best results. For peptide LFDNAMLR, summation of three product ions y_5, y_6 and y_7 was needed at MEW 0.01 Da to obtain optimal results. Because of their higher levels, for all internal standards an MEW of 0.01 Da was sufficient and summation of ions was not necessary. Table 3 shows the results for accuracy and precision found for all three peptides, obtained using the optimized settings for MEW and summation, for concentrations across the anticipated relevant concentration range. It demonstrates that the usual acceptance criteria for LC–MS as applied to small-molecule quantitation can be met.

As shown in Figure 2B-3, at low values for the MEW the mass accuracy of the detector becomes very important, since a slight shift in the mass will easily result in a major difference in the acquired signal or, in the worst case,

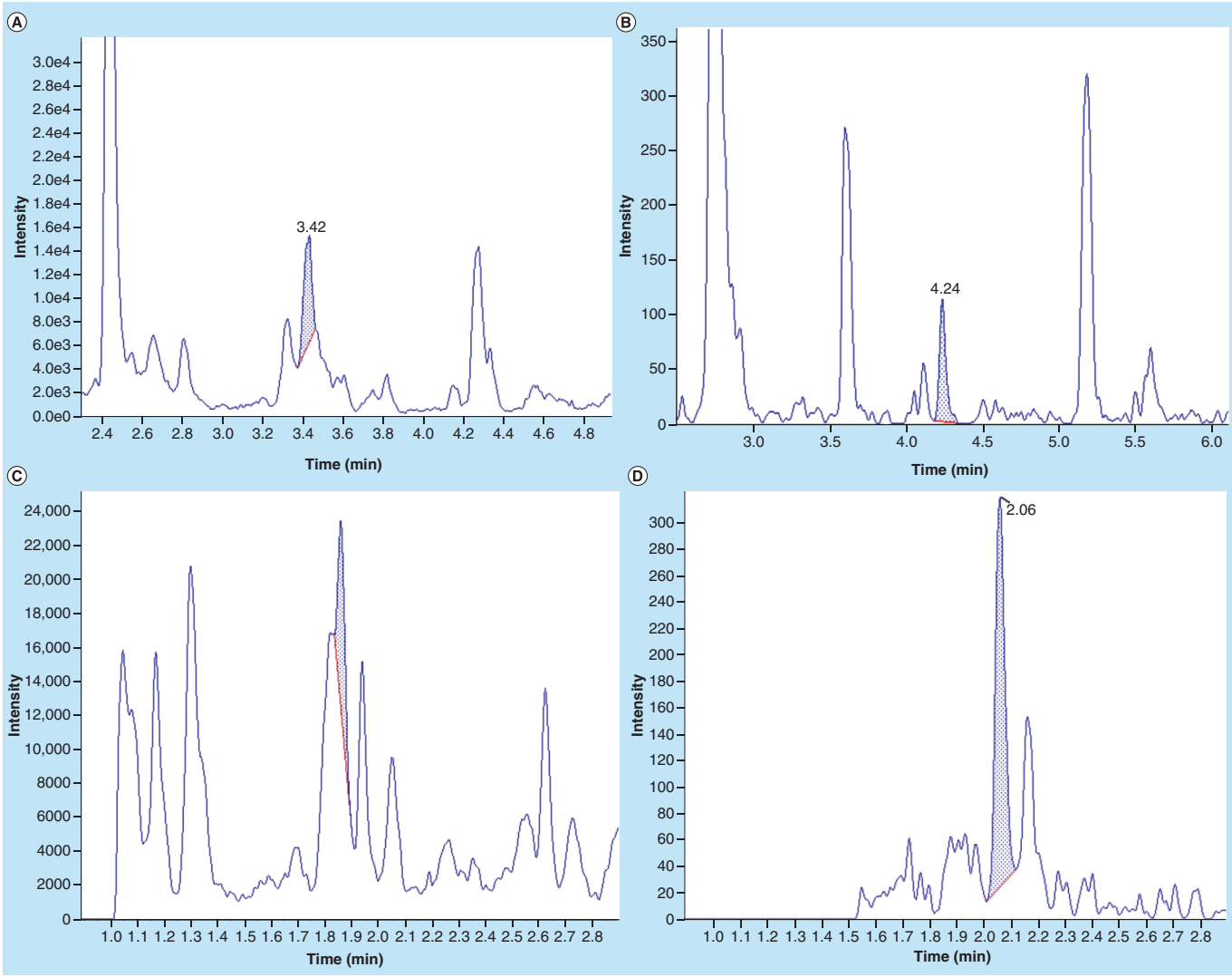

Figure 4. Chromatograms for two signature peptides after digestion of rat plasma spiked with somatropin at 25 ng/ml. (A) LC–MS/MS and **(B)** LC–HR-MS/MS for SNLELLR, **(C)** LC–MS/MS and **(D)** LC–HR-MS/MS for FDTNSHNDDALLK, recorded using the final settings as summarized in Tables 3 and 4.

in completely missing the signal. It is therefore essential that the Q-TOF system is set in a stable climate, because temperature fluctuations can influence the mass calibration due to contraction and expanding of the TOF detector tube. To ensure optimal performance, it is good practice to calibrate the system once every 2–3 h, in our situation by infusing a calibrant solution into the source after every six samples. This resulted in an excellent mass accuracy (<2 mDa) as shown in Supplementary Figure 1. Depending on the instrument type (e.g., orbitrap) and vendor, the required frequency of recalibration may be different.

Comparison to MS/MS

In Figure 4, chromatograms are shown for the peptides SNLELLR and FDTNSHNDDALLK, recorded upon triple quadrupole LC–MS/MS (Figure 4A & C) and LC–HR-MS/MS (Figure 4B & D) analysis of the same tryptic digest of rat plasma spiked with somatropin at 25 ng/ml. For LC–HR-MS/MS, the optimized settings with regard to MEW and summation of ions were used, while for LC–MS/MS the typical approach was followed of quantifying a single product ion (optimized with regard to sensitivity and selectivity) at unit–mass resolution (MEW 0.7 Da). The chromatograms confirm that LC–MS/MS generally gives rise to more interferences in the chromatograms and that a lower LLOQ can be attained using LC–HR-MS/MS, although the absolute instrument sensitivity for LC–MS/MS is better. It is also evident that the degree of improvement varies from peptide to peptide,

Table 4. LLOQ, summary of precision (CV) and accuracy results for the final LC–MS/MS settings for all three peptides; n = 6.

Peptide	Precursor ion (m/z)	Product ions (m/z)	Concentration (ng/mL)	CV (%)	Accuracy (%)
FDTNSHNDDALLK	497.2	671.8 (y_{12})	25	4.4	242.2
			50	4.5	143.6
			75	2.2	144.9
			100	2.1	94.7
			1250	0.8	93.7
			8000	0.8	107.1
SNLELLR	422.8	643.4 (y_5)	25	8.1	230.4
			50	7.3	135.8
			75	3.5	125.3
			100	5.3	87.5
			1250	0.4	84.0
			8000	0.9	94.2
LFDNAMLR	490.3	719.4 (y_6)	25	3.5	226.9
			50	4.5	131.6
			75	2.5	133.4
			100	0.9	93.0
			1250	0.5	86.6
			8000	1.0	100.2

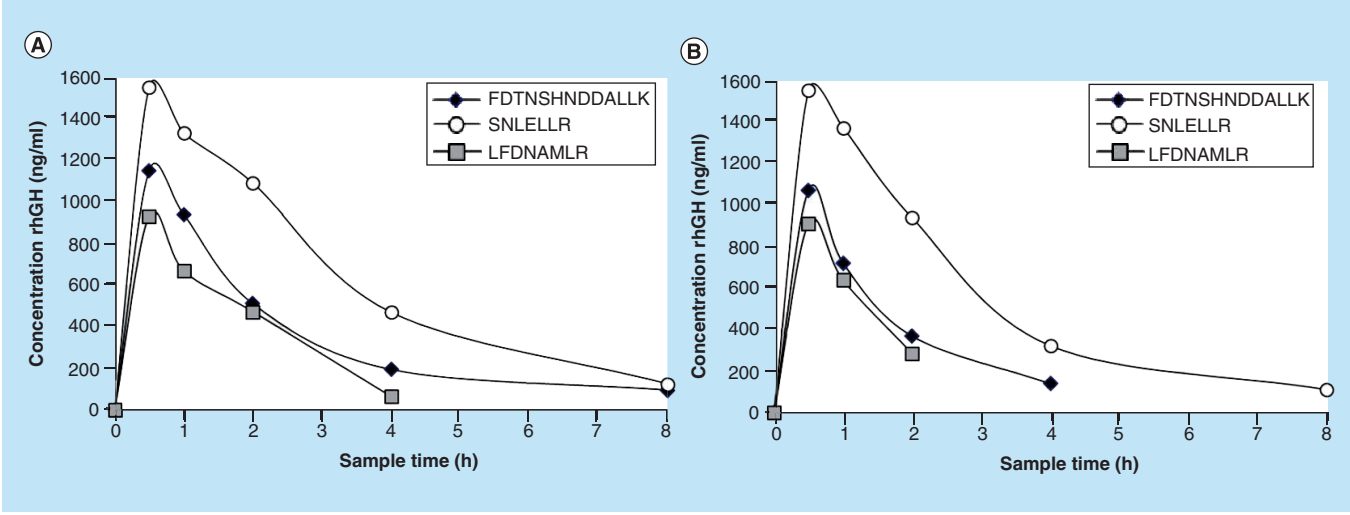

Figure 5. Somatropin (recombinant human growth hormone) plasma concentration-time curves. These were obtained for the signature peptides SNLELLR (○), FDTNSHNDDALLK (♦) and LFDNAMLR (□) after 2 mg/kg subcutaneous dosing to a rat, obtained with LC–HR-MS/MS **(A)** and LC–MS/MS **(B)**.

as FDTNSHNDDALLK suffers more from interfering matrix peptides with LC–MS/MS than SNLELLR. The resulting values for accuracy and precision that were obtained for LC–MS/MS are presented in Table 4. The interferences are clearly causing poor results for accuracy at lower somatropin concentrations, just as was observed for LC–HR-MS/MS at a MEW of 0.5 Da. Acceptable results for all three peptides were found for somatropin levels at and above 100 ng/ml, which was therefore the practical LLOQ for LC–MS/MS.

Analysis of preclinical samples

As an example, the pharmacokinetic curves for the three signature peptides, obtained by LC–HR-MS/MS analysis of plasma samples after subcutaneous dosing of somatropin to a rat, are shown in Figure 5A. These curves illustrate that the obtained LLOQ of 25 ng/ml for all signature peptides is adequate to monitor the relevant plasma somatropin concentrations. In Figure 5B, the corresponding curves after analysis of the same samples by LC–MS/MS are presented. Comparison of the two curves indicates that for some of the later time-points unquantifiable concentrations were found by LC–MS/MS because of its higher LLOQ, and that the use of HR–MS/MS for detection therefore is an advantage. Otherwise, the pharmacokinetic profiles agreed very well.

For completeness, the individual plasma concentration results for all animals are shown in the Supplementary Information (Figure 2).

The three signature peptides consistently gave dissimilar concentration results in the plasma samples of dosed rats, whereas no differences were seen in spiked samples. Generally, the highest concentrations were found for the mid-protein peptide SNLELLR. The other two peptides, which are located closer to the C- and N-terminus of somatropin, respectively, typically yielded lower and also more comparable concentrations. Altogether, this suggests an *in vivo* effect, in which the central part of the protein is less susceptible to enzymatic and/or chemical modifications than the C- and N-terminal parts. The fact that the concentrations found for FDTNSHNDDALLK and for the methionine-containing peptide LFDNAMLR generally were quite similar suggests that oxidation of the methionine probably plays a negligible role.

Discussion & conclusion

In this work, we show the potential of LC–HR-MS/MS for the quantitation of proteins after the digestion of a complex biological matrix. The use of a Q-TOF mass spectrometer with a mass resolution of about 20,000 allowed the selection of a relatively large part of the product ions that were formed after collision-induced dissociation in a narrow MEW. In this way, a good analyte detection signal was obtained, while at the same time peptides originating from the digestion of matrix proteins were largely excluded from detection. By reducing the MEW from 0.5 to 0.01 Da, the number of interfering peaks in the extracted ion chromatograms was considerably reduced which facilitated the determination of three different signature peptides. Detection sensitivity was further enhanced by the summation of the responses of multiple product ions for each of the signature peptides, allowing quantitation with acceptable accuracy and precision down to levels corresponding to 25 ng/ml of the intact protein somatropin in rat plasma.

The conditions for optimal selectivity and sensitivity varied from peptide to peptide and, in general, the MEW settings as well as the summation of responses have to be optimized for any given signature peptide and product ion. To avoid missing the detection of part or all of the product ions when a narrow MEW is used, a high mass accuracy of the mass spectrometer is important and frequent calibration of the instrument is advisable. Comparison of LC–HR-MS/MS with LC–MS/MS showed that, while the absolute instrument sensitivity of the latter typically is better, the possibility of removing interfering peaks from the chromatograms by narrowing the MEW on HRMS may finally result in lower LLOQ values. Published LC–MS methods for the quantification of somatropin or endogenous hGH after digestion use elaborate sample preparation strategies such as immunocapture and multistage SPE, in combination with nano-LC or 2D LC to achieve LLOQs in the 2.7–10 ng/ml-range with sample volumes of 100–800 µl of plasma/serum [30–32]. The results of the current work demonstrate that LC–HR-MS/MS can be a straightforward alternative to reach the same order of sensitivity. Although HRMS may not always outperform triple quadrupole MS in terms of selectivity and sensitivity, the results in the current paper show that the use of HRMS can be a useful tool to improve the performance of bioanalytical LC–MS methods for proteins.

Future perspective

With LC–MS now being an established analytical platform for protein quantitation, mass spectrometric approaches other than MS/MS on a triple quadrupole are beginning to be explored. Because of its potential to improve selectivity and sensitivity of bioanalytical methods, as shown in this paper, we expect that HRMS for protein quantitation will grow in importance in the next few years and that the technique will find its place in research and regulated laboratories, probably next to triple quadrupole MS. If technological advances continue to provide more sensitive, robust and affordable instruments, LC–HR-MS/MS may in the end be expected to become the technique of first choice for protein quantitation in complex biological matrices.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2018-0032

Summary points

- In HRMS, the mass extraction window can be reduced from 0.5 Da, which is comparable to triple quadrupole MS detection, down to values as low as 0.001 Da.
- Reducing the mass extraction window typically results in an increase of selectivity and although reduction to values below 0.05 Da will result in a loss of absolute sensitivity, there may be a gain in signal to noise ratio.
- With HRMS, it is relatively easy to sum the responses of different ions to enhance sensitivity, although this summation may also increase the response of background interferences.
- LC with HR–MS/MS detection may be a useful tool for the quantitation of proteins after digestion of biological samples and improve method selectivity and sensitivity without the need for lengthy experimental extraction procedures.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Li F, Fast D, Michael S. Absolute quantitation of protein therapeutics in biological matrices by enzymatic digestion and LC–MS. *Bioanalysis* 3(21), 2459–2480 (2011).
2. Van den Broek I, Niessen WMA, van Dongen WD. Bioanalytical LC–MS/MS of protein-based biopharmaceuticals. *J. Chromatogr. B* (929), 161–179 (2013).
3. Yang Z, Li W, Smith HT, Tse FLS. LC–MS bioanalysis of proteins. In: *Handbook of LC–MS Bioanalysis.* Li W, Zhang J, Tse FLS (Eds). John Wiley & sons, NJ, USA, 601–605 (2013).
4. Cross TG, Hornshaw MP. Can LC and LC–MS ever replace immunoassays? *J. Appl. Bioanal.* 2(4), 108–116 (2016).
5. An B, Zhang M, Johnson RW, Qu J. Surfactant-aided precipitation/on-pellet-digestion (SOD) procedure provides robust and rapid sample preparation for reproducible, accurate and sensitive LC/MS quantification of therapeutic protein in plasma and tissues. *Anal. Chem.* 87(7), 4023–4029 (2015).
6. Bronsema KJ, Bischoff R, Pijnappel WWMP, van der Ploeg AT, van de Merbel NC. Absolute quantification of the total and antidrug antibody-bound concentrations of recombinant human α-glucosidase in human plasma using protein G extraction and LC–MS/MS. *Anal. Chem.* 87(8), 4394–4401(2015).
7. Bults P, Bischoff P, Bakker H, Gietema JA, van de Merbel NC. LC–MS/MS-based monitoring of *in vivo* protein biotransformation: quantitative determination of trastuzumab and its deamidation products in human plasma. *Anal. Chem.* 88(3), 1871–1977 (2016).
8. Bronsema KJ, Bischoff R, van de Merbel NC. High-sensitivity LC–MS/MS quantification of peptides and proteins in complex biological samples: the impact of enzymatic digestion and internal standard selection on method performance. *Anal. Chem.* 85(20), 9528–9535 (2013).
9. Bischoff R, Bronsema KJ, van de Merbel NC. Analysis of biopharmaceutical proteins in biological matrices by LC–MS/MS – I. Sample preparation. *Trends Anal. Chem.* 48, 41–51 (2013).
10. Wilffert D, Bischoff R, van de Merbel NC. Antibody-free workflows for protein quantification by LC–MS. *Bioanalysis* 7(6), 763–779 (2015).
11. *Sample Preparation in Quantitative LC–MS Bioanalysis.* Li W, Jian W, Fu Y (Eds). John Wiley & Sons, NJ, USA (2013) (In Press).
12. Wilffert D, Donzelli R, Asselman A *et al.* Quantitative antibody-free LC–MS/MS analysis of sTRAIL in sputum and saliva at the sub-ng/mL level. *J. Chromatogr. B* 1032, 205–210 (2016).
13. Zhou S, Vazvaei F, Ferrari L, Qu J. Practical considerations in enhancing LC–MS sensitivity for therapeutic protein bioanalysis. *Bioanalysis* 9(18), 1353–1356 (2017).
14. Huang MQ, Lin Z, Weng N. Applications of high-resolution MS in bioanalysis. *Bioanalysis* 5, 1269–1276 (2013).

15. Fung EN, Jemal M, Aubry AF. High-resolution MS in regulated bioanalysis: where are we now and where do we go from here? *Bioanalysis* 5, 1277–1284 (2013).
16. Zhang NR, Yu S, Tiller P, Yeh S, Mahan E, Emary WB. Quantitation of small molecules using high-resolution accurate mass spectrometers – a different approach for analysis of biological samples. *Rapid Commun. Mass Spectrom.* 23, 1085–1094 (2009).
17. Henry H, Sobhi HR, Scheibner O, Bromirski M, Nimkar SB, Roc B. Comparison between a high-resolution single-stage Orbitrap and a triple quadrupole mass spectrometer for quantitative analyses of drugs. *Rapid Commun. Mass Spectrom.* 26, 499–509 (2012).
18. Dillen L, Cools W, Vereyken L *et al.* Comparison of triple quadrupole and high-resolution TOF-MS for quantification of peptides. *Bioanalysis* 4, 565–579 (2012).
19. Morin LP, Mess JN, Garofolo F. Large-molecule quantification: sensitivity and selectivity head-to-head comparison of triple quadrupole with Q-TOF. *Bioanalysis* 5, 1181–1193 (2013).
20. Plumb RS, Fujimoto G, Mather J *et al.* Comparison of the quantification of a therapeutic protein using nominal and accurate mass MS/MS. *Bioanalysis* 4, 605–615 (2012).
21. Mekhssian K, Mess JN, Garofolo F. Application of high-resolution MS in the quantification of a therapeutic monoclonal antibody in human plasma. *Bioanalysis* 6, 1767–1799 (2014).
22. Kellie JF, Kehler JR, Szapacs ME. Application of high-resolution MS for development of peptide and large-molecule drug candidates. *Bioanalysis* 8, 169–177 (2016).
23. Sundberg M, Strage EM, Bergquist J, Holst BS, Ramström M. Quantitative and selective analysis of feline growth related proteins using parallel reaction monitoring high resolution mass spectrometry. *PLoS ONE* 11(12), e0167138 (2016).
24. Sun H, Zhang Q, Zhang Z, Tong J, Chu D, Gu J. Simultaneous quantitative analysis of polyethylene glycol (PEG), PEGylated paclitaxel and paclitaxel in rats by MS/MSALL technique with hybrid quadrupole time-of-flight mass spectrometry. *J. Pharm. Biomed. Anal.* 145, 255–261 (2017).
25. Zhao Y, Liu G, Yuan X *et al.* Strategy for the quantitation of a protein conjugate via hybrid immunocapture-liquid chromatography with sequential HRMS and SRM-based LC–MS/MS analyses. *Anal. Chem.* 89(9), 5144–5151 (2017).
26. Niedermeyer THJ, Strohalm M. mMass as a software tool for the annotation of cyclic peptide tandem mass spectra. *PLoS ONE* 7(9), e44913 (2012)
27. Altschul S, Madden T, Schaffer A *et al.* Gapped BLAST and PSI- BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25(17), 3389–3402 (1997).
28. Bults P, van de Merbel NC, Bischoff R. Quantification of biopharmaceuticals and biomarkers in complex biological matrices: a comparison of liquid chromatography coupled to tandem mass spectrometry and ligand binding assays. *Expert Rev. Proteomics* 12(4), 355–374 (2015).
29. Campbell JL, Le Blanc JC. Peptide and protein drug analysis by MS: challenges and opportunities for the discovery environment. *Bioanalysis* 3(6), 645–657 (2011).
30. Pritchard C, Groves KJ, Biesenbruch S *et al.* Quantification of human growth hormone in serum with a labeled protein as an internal standard: essential consideration. *Anal. Chem.* 86(13), 6525–6532 (2014).
31. Arsene CG, Henrion A, Diekmann N *et al.* Quantification of growth hormone in serum by isotope dilution mass spectrometry. *Anal. Biochem* 401(2), 228–235 (2010).
32. Such-Sanmartín G, Bache N, Bosch J *et al.* Detection and differentiation of 22 kDa and 20 kDa growth hormone proteoforms in human plasma by LC–MS/MS. *Biochim. Biophys. Acta* 1854(4), 284–290 (2015).

Special Report
10th Anniversary Issue of *Bioanalysis*

Bioanalysis

Protein quantification by LC–MS: a decade of progress through the pages of *Bioanalysis*

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Over the past 10 years, there has been a remarkable increase in the use of LC–MS for the quantitative determination of proteins, and this technique can now be considered an established bioanalytical platform for the quantification of macromolecular drugs and biomarkers, next to the traditional ligand-binding assays. Many researchers have contributed to the field and helped improve both the technical possibilities of LC–MS-based workflows and our understanding of the meaning of the results that are obtained. As a tribute to *Bioanalysis*, which has published many important contributions, this report gives a high-level overview of the most important trends in the field of protein LC–MS, as published in this journal since its inauguration a decade ago. It describes the major technical developments with regard to sample handling, separation and MS detection of both digested and intact protein analysis. In addition, the relevance of the complex structure and *in vivo* behavior of proteins is discussed and the effect of protein–protein interactions, biotransformation and the occurrence of isoforms on the analytical result is addressed.

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Over the past decade, since the inception of this journal, there have been many developments in the field of bioanalysis, but one of the most prominent probably is the rise of LC–MS for the quantification of proteins. While the determination of macromolecular biomarkers and biopharmaceuticals typically was the realm of ligand-binding assays (LBAs) 10 years ago, with LC–MS mainly being used for small molecules, it is fair to say that LC–MS has now also become an established platform for protein quantification. Because of its fundamentally different analytical principles, LC–MS can provide qualitative and quantitative information about proteins in biological samples that is complementary to LBA results. As such, it can contribute significantly to understanding the *in vivo* fate of dosed or endogenous macromolecules, whose structure and properties are so much more complex than those of traditional drugs and biomarkers of low molecular weight. In some cases, LC–MS may even be the only technique capable of providing a useful quantitative result.

Since the first quantitative bioanalytical LC–MS methods for proteins started to be reported, a little over 10 years ago, many scientists have contributed to the development of the field. LC–MS-based bioanalysis for proteins is different in many ways from small-molecule LC–MS as well as from LBAs, so much needed to be learned. Understandably, developments started with a clear focus on the technological aspects and much effort has gone into optimizing and properly combining different analytical tools to allow quantification of proteins with the required sensitivity, selectivity, accuracy and precision. While with a well-developed LC–MS assay for a small molecule, there generally is no discussion about what a concentration result represents, it was soon realized that this is not so straightforward for large and structurally complex proteins. LC–MS, like most LBAs, usually targets only a part of the analyte, and the concentration result obtained is strongly dependent on the exact part of the protein to which the method is directed and on the underlying analytical principle of the assay. Consequently, the field of protein bioanalysis has seen increasing attention for what a concentration result, generated by LC–MS, exactly represents.

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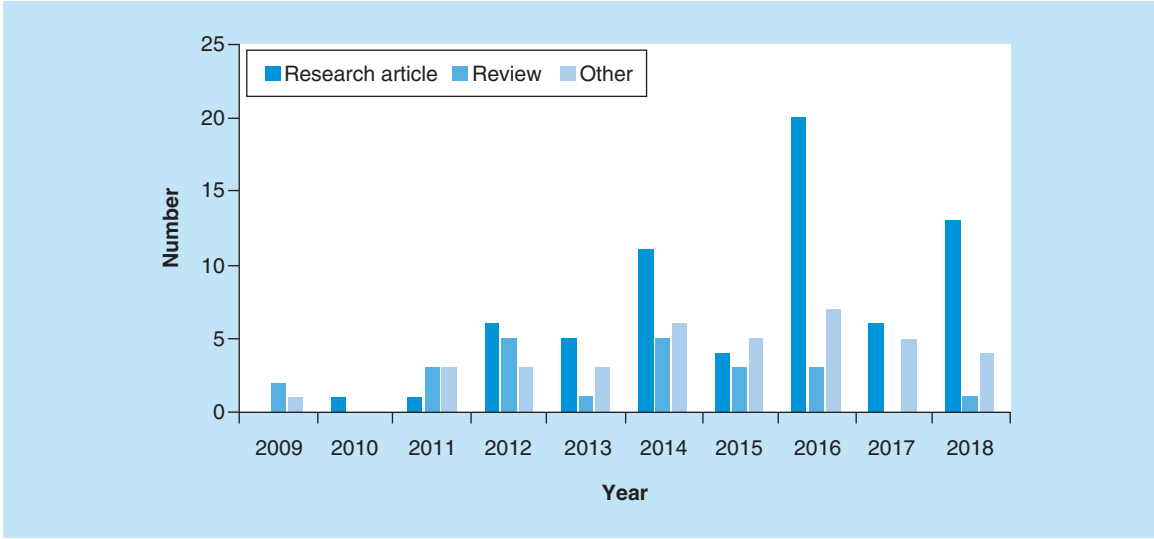

Figure 1. Historical overview of research papers, reviews and other contributions (editorials, perspectives, commentaries) on protein LC–MS, as published in *Bioanalysis* since 2009.

Many important contributions in the area of LC–MS-based protein quantification have been published in *Bioanalysis* (Figure 1). As a tribute to the 10-year anniversary of the journal, this report gives a high-level overview of the most important developments and trends in the field, which have appeared on the pages of *Bioanalysis* in the past decade. This overview is by no means meant as an exhaustive review of the literature, because many key papers have obviously also appeared in other journals. Among the many papers published in this journal, there are several excellent reviews of different aspects of this broad field, to which the interested reader is referred for more detailed information.

The analysis

Digested protein analysis

The traditional approach of protein quantification by LC–MS was, and often still is, the enzymatic digestion of a protein analyte into a mixture of peptides and the quantification of one or a few of these as a surrogate readout for the intact protein. The advantages are obvious. Large proteins are typically difficult to quantify by the classical combination of a reversed-phase LC column and a triple-quadrupole mass spectrometer. Enzymatic digestion reduces the complicated analyte into a much more manageable set of smaller peptides, and their quantification can be based on the decades of accumulated knowledge that we have of small-molecule and, especially, peptide LC–MS. At the same time, protein quantification can readily benefit from the general technical advantages of LC–MS, such as a wide linear dynamic range, good accuracy and precision because of the ability to apply internal standards, the possibility of quantifying multiple analytes simultaneously and the independence of critical immunoreagents of potentially poor quality.

Digestion

Not surprisingly, there was and is much enthusiasm in the bioanalytical community, and notably among LC–MS scientists, about the potential of this technique for protein quantification. Several review papers were published in this journal on the technical aspects of targeted LC–MS quantification of proteins as early as 2011 [1–3]. They emphasized the importance of the digestion step as the central part of the workflow, and the selection of a proper surrogate peptide for quantification, but otherwise approached protein bioanalysis essentially as an extension of peptide bioanalysis. Developments have been rather limited, when it comes to the digestion step. By far most researchers are using trypsin as the digestion enzyme of first choice [4]. This is mainly due to its wide availability for a reasonable price and its ability to cleave proteins into peptides of a size that is compatible with LC–MS/MS analysis on a triple quadrupole. If no suitable peptides are formed by trypsin, alternative enzymes such as chymotrypsin [5] or Glu-C [6] can also be used, or even chemical digestion, such as in the case of the presence of an acid-labile amide bond in the protein of interest [7].

Plasma or serum can be directly digested in solution [1–3], but it is very useful to first isolate the protein fraction from the sample by protein precipitation and centrifugation, and then resuspend and digest the obtained protein pellet. This approach, known as pellet digestion [8,9], has the advantage that it concentrates the sample and that some matrix interferences, such as smaller proteins and phospholipids, are removed. Traditionally, to ensure its completeness, digestion is often performed overnight, which makes protein quantification a 2-day process. Although undesirable from a sample throughput perspective, it is an approach that is still used in many recent reports, even though digestion times can be reduced to a few hours, if properly optimized, and even to a matter of minutes when using an immobilized trypsin reactor [10,11]. An interesting innovation worth mentioning is the concept of limited proteolysis, which was reported for the quantification of a monoclonal antibody (mAb) [12]. In this approach, the protein analyte is selectively captured within the relatively small pores of a resin, while trypsin is immobilized on a larger particle, which cannot completely penetrate into the pore. In this way, only a small part of the analyte is digested, which reduces the complexity of the digest.

Most published digestion protocols include denaturation, reduction and alkylation steps, prior to digestion, to unfold the protein structure and break intramolecular disulfide bonds. This typically makes the inner part of a protein more accessible to the digestion enzyme and will speed up the digestion process, but these steps do not necessarily improve the overall analysis. If the surrogate peptide is located at the protein surface and does not contain a disulfide-forming cysteine it may be beneficial to skip those steps, to reduce the formation of interfering peptides originating from endogenous proteins [13].

Protein & peptide enrichment

Despite all advantages, it was also evident that LC–MS suffers from a major technological drawback compared with an LBA: its relatively limited sensitivity, and much scientific effort has gone into addressing and improving this situation. An important reason for the limited sensitivity of LC–MS for protein bioanalysis is the high abundance of endogenous proteins in complex matrices such as serum and plasma. If these proteins are all also digested, an even more complex mixture is formed, containing of a myriad of peptides with very similar analytical properties. Many of these peptides have the same or nearly the same mass, and the same fragmentation behavior, as the surrogate peptide and show up as interferences in the chromatograms. In this way, the matrix background can be responsible for a reduction of more than a factor 100 in the achievable LLOQ [14]. Consequently, for matrices with a lower endogenous protein content, such as bronchoalveolar lavage fluid, obtaining a good sensitivity is usually much easier [15].

The enrichment of the protein analytes from the biological matrix and/or the surrogate peptides from the digest is an obvious way to improve sensitivity and much research has been devoted to this over the years [16]. As reviewed in 2015 [17], a variety of generic enrichment methods have been developed, most successfully if the protein analyte is structurally different from the bulk of the endogenous matrix proteins. PEGylated proteins, for example, can easily be enriched by extraction into an organic solvent. Solid-phase extraction on ion-exchange materials is a possibility if a protein's [18] or peptide's [19] isoelectric point is clearly different from that of their endogenous counterparts, while molecules containing histidine moieties on their surface can be isolated by complexation with immobilized metal ions, such as nickel, an approach called immobilized metal affinity chromatography [20,21]. By applying one or a combination of these techniques, protein quantification in complex biological matrices down to low- to sub-ng/ml level has been achieved, but a good knowledge of the analyte's properties is essential and there is no guarantee that the required sensitivity can indeed be reached.

Affinity capture

If more selectivity and sensitivity are needed, enrichment based on analyte-specific affinity interaction often is the better option [22,23]. It should be kept in mind, however, that the reagents needed for affinity capture in principle suffer from the same disadvantages as those used for LBAs, such as batch-to-batch differences and limited availability, which could remove one of the advantages of using LC–MS over LBA. Still, many researchers appear to have ready access to reagents of sufficient quality and several published LC–MS methods for proteins include an affinity capture step. Affinity-based enrichment can also be quite generic, such as the use of protein A or protein G to capture therapeutic antibodies of the IgG type from a biological sample [24], although it is a disadvantage that the very abundant and heterogeneous endogenous IgG fraction in the plasma of many species also binds to protein A/G. A more selective alternative is affinity capture using an antibody directed to the constant (Fc) part of human IgG to selectively isolate a variety of human or humanized mAbs from animal matrices [25,26]. Like in

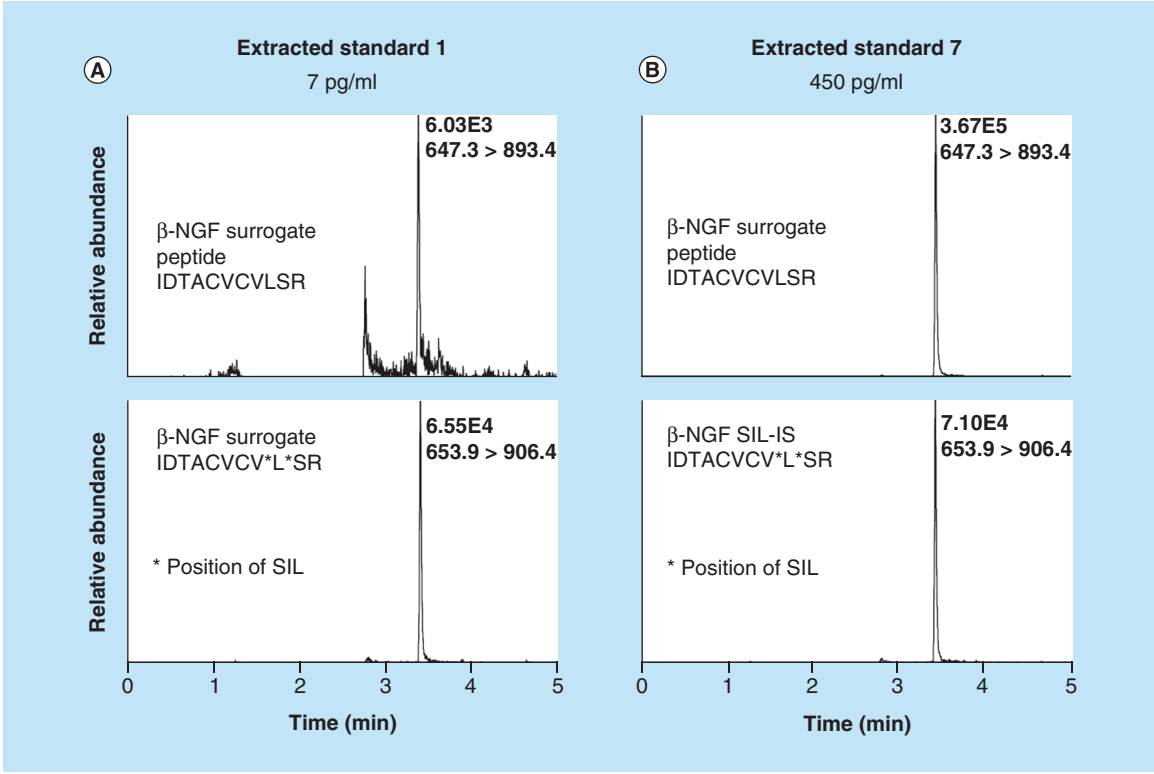

Figure 2. LC–MS/MS chromatograms of the surrogate peptide and the corresponding internal standard of β-nerve growth factor. LLOQ standard at 7 pg/ml **(A)** and ULOQ standard at 450 pg/ml. Protein extracted from plasma and peptide extracted from digest by immunocapture.
Reproduced from [36] © Future Science Group (2016).
SIL: Stable-isotope labeled.

LBAs, ultimate selectivity is obtained with a capturing reagent that is specifically directed to the analyte of interest, for example, an anti-idiotypic antibody [27] or the pharmacological target [28]. In this way, sensitivity down to the pg/ml level can typically be reached.

Recently, alternative materials have emerged for affinity capture. Aptamers, for example, are oligonucleotide molecules [29] and affimers are engineered polypeptides [30], that both bind to proteins with high affinity and have been used for quantitative bioanalysis of proteins. In addition, synthetic molecular imprinted polymers (MIPs) can also extract proteins from biological samples and offer a further diversification of affinity capture approaches [31]. While most researchers use reagents immobilized on magnetic beads, which are mixed with the sample and subsequently collected by applying a magnet, other formats have also been developed and successfully used. Examples include the immobilization of a capturing agent in the wells of an ELISA plate [32] and on a monolithic material present in a pipette tip, which aspirates and dispenses the sample multiple times [33]. Affinity capture is usually performed on the intact protein analyte before digestion but can equally well be done after digestion. In that case, a capture reagent is used which selectively extracts the surrogate peptide(s) from the digest [34,35]. In case extreme sensitivity is needed, both pre- and postdigestion affinity enrichment can be combined, as was convincingly demonstrated for the biomarker β-NGF (Figure 2). By capturing the protein with a polyclonal antibody immobilized on magnetic beads, digesting it with trypsin and subsequently capturing the surrogate peptide on a column containing an antipeptide antibody, concentrations down to 7 pg/ml could be routinely quantified [36].

Liquid chromatography

After digestion of a protein analyte and further purification of the sample, the actual analysis typically is not much different from standard small-molecule quantification. Reversed-phase LC in combination with positive-mode electrospray tandem mass spectrometry is the gold standard. Run times tend to be somewhat longer than for small

molecules, especially if the digest has a complex composition and separation of the surrogate peptide from interfering matrix peptides is needed. Ultra-high-performance LC columns with sub-2 µm particles are increasingly being used to decrease analysis time and/or improve resolution [37]. If sensitivity is not limited by matrix interferences, lower detection limits can generally be obtained by analyzing the same volume of processed sample on a column with a reduced diameter and a lower mobile-phase flow rate [38]. Technical advances in this field of micro- or nano-LC were reviewed in 2014 and it was concluded that the benefits are most effectively realized in combination with a thorough sample cleanup such as immunodepletion and immunoaffinity enrichment [39]. The use of special equipment with extremely low dead volumes is essential, but robust commercial systems are now widely available. The high separation power of LC has led some researchers to use this technique as a sample preparation tool. By collecting the mobile-phase fraction containing the surrogate peptide and injecting it into a second LC system with complementary selectivity, additional cleanup can be achieved, albeit at the expense of a limited sample throughput [40,41]. Opportunities for coupling these two LC steps on-line in a 2-D system are there [42] but have certainly not been applied on a large scale.

Without doubt, the possibility to determine several compounds in a single run is a very attractive feature of LC–MS in comparison with the typical LBA, although the majority of published methods is still restricted to a single analyte. The simultaneous quantification of two biopharmaceuticals in plasma, given as a combination therapy [43] or dosed together for research purposes [18], has been reported, as well as the measurement of a biopharmaceutical protein together with its pharmacological (soluble) target [44]. The LC–MS quantification of two appropriate surrogate peptides for such a combo-assay is relatively straightforward, but special attention might be needed to simultaneously extract the two protein analytes from the matrix, for example, by two different affinity capture reagents.

Mass spectrometry

Surrogate peptides can robustly be quantified on our bioanalytical workhorse, the triple-quadrupole mass spectrometer, by detection of a peptide fragment after collision-induced fragmentation in the multiple reaction monitoring (MRM) mode. Depending on its size and structure, a peptide may form ions with several charge states, but one of these usually dominates in terms of intensity and is, thus, selected for quantification. If needed, the responses of multiple peptide ions can be summated for improved sensitivity.

A recent development is the introduction of high-resolution mass spectrometry (HRMS) for quantitative bioanalysis, usually in the form a quadrupole-time of flight (Q-TOF) instrument. The main advantage of HRMS for the quantification of surrogate peptides in a complex digest is its improved ability to separately detect the responses of molecules with very close molecular masses, and therefore provide a more selective detection of a surrogate peptide. By narrowing the mass extraction window from 0.7 Da, the typical value for a triple quadrupole, down to, for example, 0.01 Da, most of the ions of a surrogate peptide of interest can still be detected, but a major part of the interferences from digested plasma proteins is no longer selected for detection. This better selectivity may, however, not always lead to better quantification limits, because the absolute instrument sensitivity of triple-quadrupole MS still is superior to that of HRMS, although the difference has decreased over the years. In a report published in 2012, LC–MS/MS quantification of a surrogate peptide on a Q-TOF MS was ten-times less sensitive than on a triple quadrupole [45], but a publication from 2018 showed a fourfold improvement of the achievable LLOQ on a Q-TOF compared with triple-quadrupole MS, even though the absolute instrument sensitivity of HRMS still was less favorable [46]. Analysis on a Q-TOF in the MRM mode (unit mass resolution in the quadrupole followed by high resolution in the TOF) is essential for a good signal to noise ratio: the LLOQ for an mAb in plasma obtained using TOF only without fragmentation of the surrogate peptide was found to be 25-times higher than for Q-TOF analysis in MRM mode [47].

An interesting and relatively unexplored area is the application of supercharging reagents to improve the sensitivity of MS detection of peptides. By adding a specific chemical such as m-nitrobenzyl alcohol to the LC mobile phase, the ionization process of a surrogate peptide (and other peptides) is changed. Generally, the absolute ionization efficiency is increased as well as the relative intensity of the more highly charged species, but background noise may also increase [48]. This approach has the potential to contribute to the improvement of the sensitivity of protein bioanalysis, although more research is clearly needed to establish its full potential.

Note

An attractive feature of digested protein analysis is the possibility to use a single peptide as a surrogate for multiple proteins. Rather than using a surrogate peptide from the variable region of an mAb, a so-called universal peptide is selected from the constant region of human IgG. Such a peptide is present in the sequence of all human therapeutic mAb (candidate) drugs but is not found in the endogenous proteins of any animal species. Thus, a single analytical method can be used to support the preclinical development of a diversity of human mAbs across multiple species, which eliminates the need for repeated separate method development [49]. Methods for seven different mAbs in serum of three different nonhuman species were set up and successfully validated in this way [50]. The approach can, unfortunately, not be used for clinical bioanalysis, because the amino acid sequence of any possible universal peptide will always occur in endogenous human IgG. The only exception was shown for a therapeutic mAb of the IgG4 subclass, which had been structurally modified to stabilize the hinge region and which contains a peptide with a sequence that does not occur in endogenous human antibodies [51].

Intact protein analysis

Over the past few years, reports have started to appear on the quantification of intact proteins by LC–MS. Despite the success of digestion-based methods, there is an increasing awareness that a surrogate peptide may represent no more than a few percent of the total protein and that, even if multiple peptides are monitored, information about most of the protein structure is lost. In an early review paper of 2015 about the quantification of intact proteins [52], a summary of the existing literature was provided. At the time, nearly all published methods were for small proteins with molecular masses below 15 kDa, but more recently much larger intact proteins have also been successfully quantified.

Mass spectrometry

While triple–quadrupole MS systems have been primarily used for surrogate peptide quantification after protein digestion, intact proteins typically need HRMS. Although the orbitrap platform provides the best mass resolution and has been applied for intact protein quantification [53], most quantitative LC–MS methods published in *Bioanalysis* use Q-TOF instrumentation. Sensitivity easily becomes an issue with increasing molecular weight, because of the distribution of the detection signal over more and more charge states, each with their own isotope distribution. The 14-kDa test protein lysozyme, for example, showed six different charge states and the summation of the most abundant isotope peak of each charge state was needed for detection down to the ng/ml range [54]. The mass spectrum of large proteins, such as mAbs (150 kDa), can be deconvoluted using dedicated software and all (typically about 30) charge states are converted into the corresponding and much simplified neutral spectrum, which is subsequently used as the basis for quantitation (Figure 3). The proof of principle of this approach was demonstrated for the determination of a mAb in plasma [55]. The summation of the three most abundant charge states of this protein showed similar selectivity, accuracy and precision as the deconvolution approach and allowed quantification down to 50 ng/ml in plasma [56]. For another mAb, the summation of responses of up to 18 different charge states resulted in better data for precision and accuracy than deconvolution [56], while for an intact antibody–drug conjugate (ADC), deconvolution gave equally acceptable results as quantification based on the response of a single-charge state [57]. For a number of mAb subunits (12–25 kDa), it was shown that the use of a limited number of charge states provided the best signal-to-noise ratio and that deconvolution can be comparable to charge state quantification but may also be worse [58].

The traditional LC–MS quantification of an intact protein occurs under denaturing conditions, which means that its 3D conformation is typically lost. Replacing settings like high organic solvent concentrations and low pH values by milder conditions allows analysis of proteins in their native state, which could be more closely related to their active form. The feasibility of quantification by native LC–MS was demonstrated for a mAb [59]. Considerably fewer charge states were observed, and the achieved sensitivity of 5 µg/ml was 25-fold less favorable than under denaturing conditions due to the suboptimal electrospray solvents, but the method was successfully validated and applied to preclinical samples.

Liquid chromatography

Developments in the chromatographic separation of intact protein species have been relatively slow over the past years. Typically, stationary phases with large pores are used at high temperatures to minimize band broadening due to slow mass transfer. Reversed-phase LC on columns packed with C4 phases with 300–1000 Å pores have

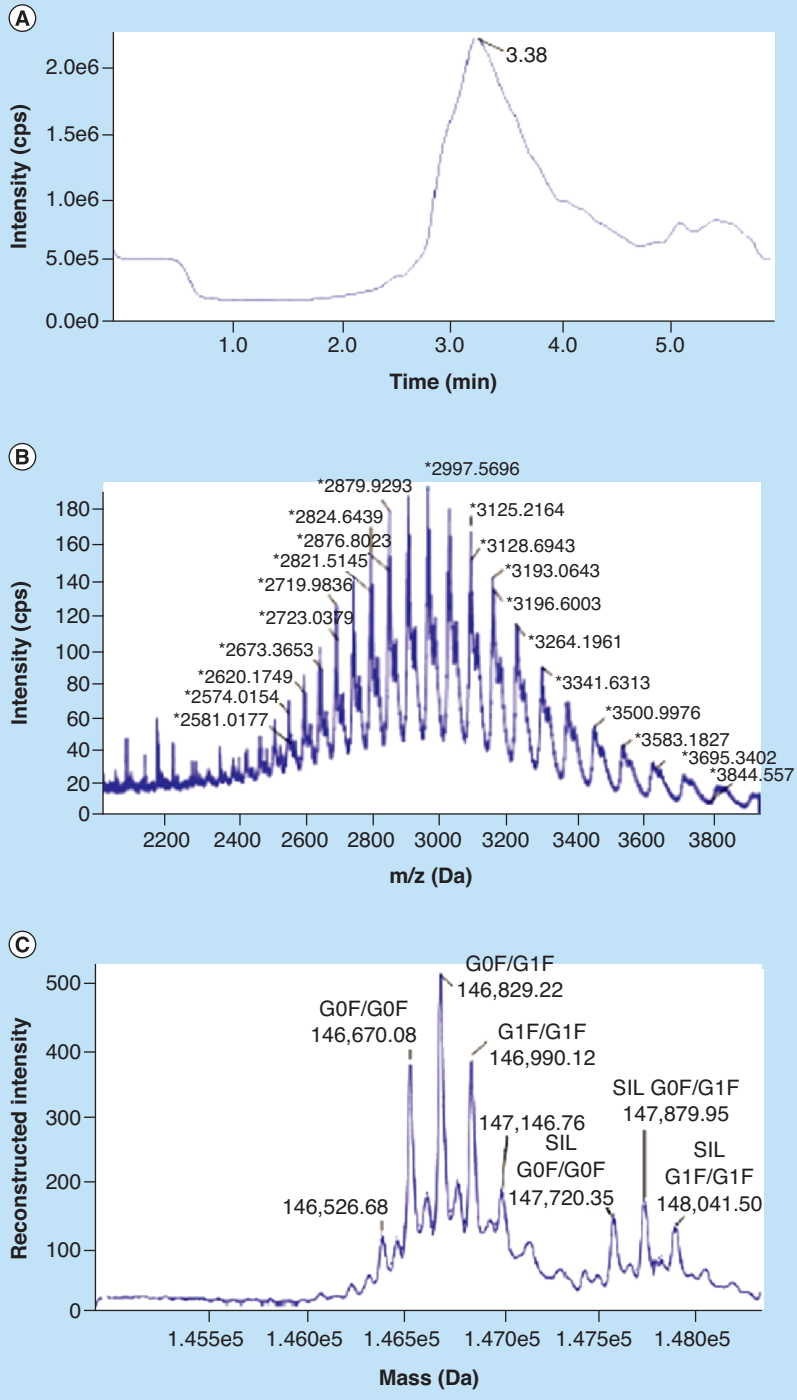

Figure 3. LC–HRMS analysis of a monoclonal antibody at 15 μg/ml in mouse plasma. (A) Chromatogram of a full TOF scan; **(B)** Raw mass spectrum for the peak at 3.38 min; **(C)** Deconvoluted mass spectrum showing multiple glycoforms (G0F and G1F) of analyte and SIL internal standard.
SIL: Stable-isotope labeled; TOF: Time of flight.
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been most widely used, in combination with a water/acetonitrile gradient in the presence of 0.1% formic acid and at a temperature of typically 70°C. Examples of bioanalytical methods for intact mAbs and ADCs on both conventional bore [54,55,57] and microbore LC [60] have been published. Alternatively, monolithic LC columns can be used for protein separation. A monolith is a single polymer rod, which contains a network of through pores and has improved mass transfer properties. As such, it is attractive for protein separation, which is inherently hampered by slow diffusion rates. A 1-mm i.d. monolithic polymer LC column was used under reversed-phase conditions in a method for the quantification of an intact mAb in plasma [56]. Native LC–MS obviously needs chromatographic conditions that keep the protein structure in its natural conformation, so reversed-phase LC with its high concentrations of organic modifiers is not ideal. A wholly aqueous, 2D LC system, in which size-exclusion and weak cation-exchange chromatography were coupled, has been described for the quantification of an mAb in plasma under native conditions [59].

Protein enrichment

LC–MS of large intact proteins needs a more thorough sample cleanup than LC–MS/MS of digested proteins, mainly because large endogenous proteins will also produce highly complex MS that will easily interfere with the analyte ions used for quantitation. Therefore, a very selective extraction of the protein analyte is essential. All published methods for quantification of an intact mAb by LC–MS include an immunocapture step, either using an antihuman IgG antibody (for analysis of animal samples) [54–57] or an anti-idiotypic antibody (for analysis of human samples) [60]. For native LC–MS, elution of the protein from the capture material has to take place under nondenaturing conditions, such as by an acetate buffer at pH 4 [59]. Cleavage of an mAb into 25-kDa fragments, by digestion with the enzyme IdeS followed by disulfide reduction, opens up the possibility to reduce the size of protein species and simplify their analysis. In this way, the glycosylation pattern of an mAb can be studied by LC–HRMS via the deconvoluted mass spectra of the different glycoforms [60].

The result

Because of their structural complexity, quantitative bioanalysis for proteins is not as straightforward as for small molecules. Usually, only a specific part of a protein molecule responds to an analytical technique and, therefore, different concentration results may be obtained for a given sample via different techniques, depending on their analytical principle and the part of the protein to which they are directed. For a meaningful concentration result, it is not sufficient to analyze samples with proper sensitivity, selectivity, precision and accuracy, but it is also important to understand what exactly the result represents [61]. Proteins often have different isoforms (including a variety of post-translational modifications), they can occur *in vivo* in free and bound form and may undergo biotransformation during their residence in the body (Figure 4) [62], and all these factors have to be considered when selecting the appropriate bioanalytical approach, be it LBA or LC–MS, for answering a particular question [63,64].

Free, bound or total concentration

Proteins can form complexes with other macromolecular binding partners *in vivo*: biopharmaceuticals will bind to their pharmacological target and/or to antidrug antibodies (ADAs), while many biomarkers have specific binding proteins. The unbound (free) and bound forms of a protein analyte exist in an equilibrium, which can easily shift when a sample is processed. Even simple dilution of a biological sample will theoretically lead to a shift of the equilibrium and a change in the free and bound concentrations [65]. The true free concentration of a protein in a complex biological matrix is, therefore, inherently difficult to establish. Methods that employ a binding step of the protein analyte, such as LC–MS with immunocapture, are often assumed to provide free concentrations, because only the free fraction is available for interaction and can be captured, but in reality the situation is more complicated. When the sample comes into contact with the binding material, the equilibrium between analyte and its endogenous binding partner is disturbed and the presence of an immobilized high-affinity binder may shift the equilibrium and result in the capture of a larger fraction. The magnitude of this fraction is difficult to predict, as it depends on the binding affinities of the protein analyte to its endogenous and capturing binding proteins, their concentrations and the time of incubation, but in the most extreme case it will be equal to the total concentration.

For a more correct estimation of the free protein concentration in a sample, the so-called free analyte quality control (QC) concept has been developed [66]. It is based on the addition of known amounts of the protein analyte and its endogenous binding partner to a QC sample that is originally free of both. Using mathematical models based on the binding affinity between the two partners, the final free analyte concentration in the QC sample

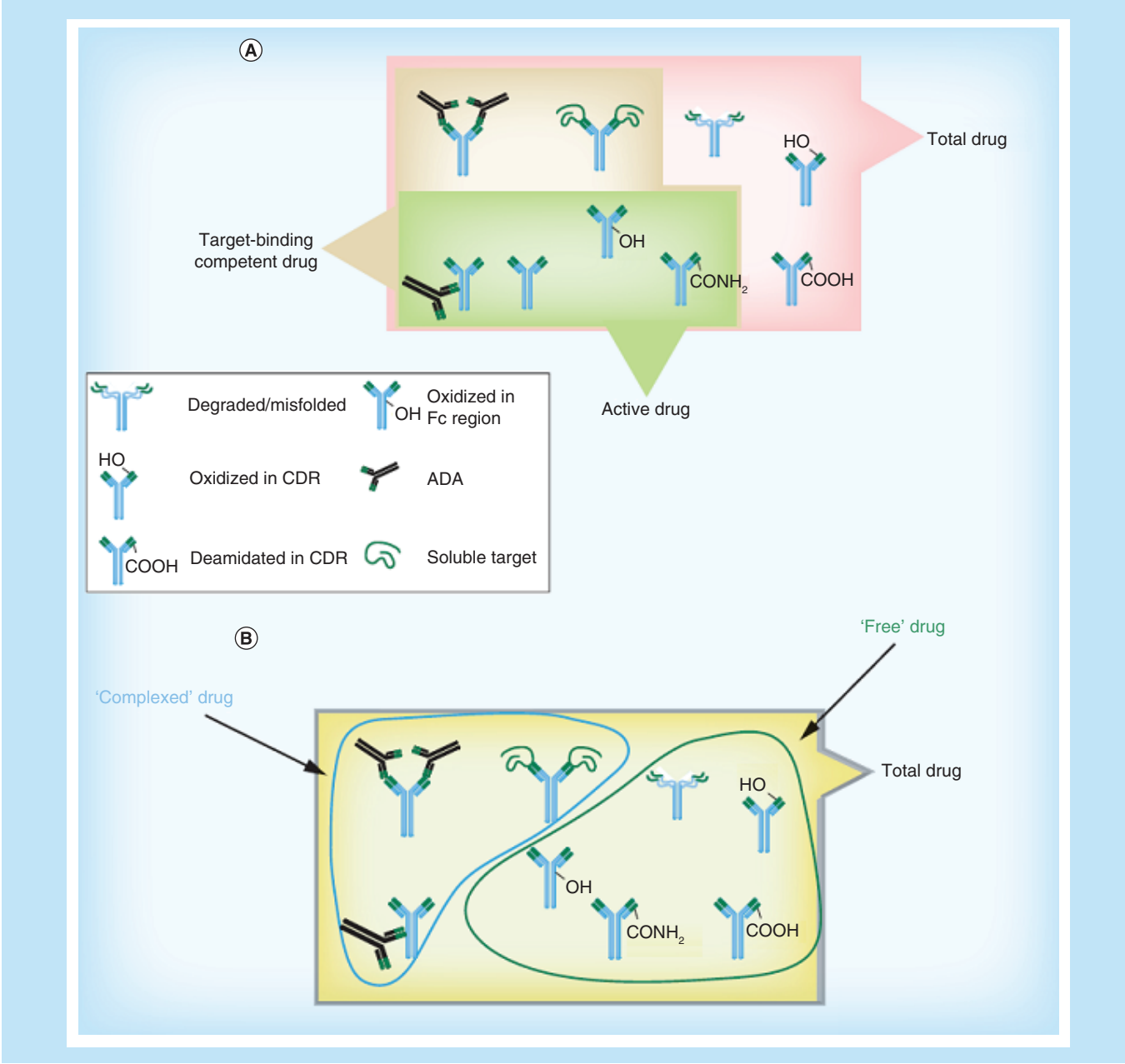

Figure 4. Occurrence of different protein forms *in vivo*, illustrated for a monoclonal antibody. Depicted are biotransformation (oxidation, deamidation, degradation) and binding (to ADA, soluble target), and its consequences for activity **(A)** and the presence or absence of drug complexes **(B)**.
ADA: Antidrug antibody.
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can be calculated. By comparison to calibrators which contain the analyte but are free from the binding protein, the free fraction is also experimentally determined. If the theoretical free fraction of the QC sample is confirmed, the assumption is that the bioanalytical method also gives correct free concentrations for any study sample. A proprietary mAb was quantified using affinity capture with the pharmacological target immobilized on magnetic beads, followed by trypsin digestion and LC–MS/MS analysis of a surrogate peptide. A 10-min incubation step of sample and beads provided free concentrations that agreed with the theoretical prediction, but overnight incubation resulted in a considerable overestimation, probably because the original equilibrium of analyte and its endogenous target had shifted [67].

Although the free concentration of a protein drug is responsible for the pharmacological effect and its determination is useful, it is practically more straightforward to determine a total concentration. Digestion of a biological sample without further protein extraction cleaves all matrix proteins into peptides and will, in principle, break all existing protein–protein interactions. Still, it is desirable to experimentally check if the digestion conditions are appropriate for quantification of the total concentration. In a report on direct digestion of trastuzumab in serum, it was observed that the addition of polyclonal ADAs resulted in a considerable negative bias of the serum concentration results [68], which suggests that the digestion conditions were insufficient to cleave the protein–ADA complex to the same extent as the free protein. The simple addition of DMSO resolved this issue, probably by making the complex more accessible to the digestion enzyme. Alternatively, an acid dissociation step will often do the job, as was demonstrated for a PEGylated therapeutic protein [13]. The addition of an equal volume of 10% formic acid to human serum before pellet digestion helped achieve correct total drug concentrations in the presence of ADAs and the soluble target.

If both free and total protein concentrations are needed, for example, to study the target engagement of a drug, complementary sample processing steps can be used. Free concentrations of the biomarker IP-10 were determined in serum by capturing this protein with an immobilized anti-IP-10 antibody that was unable to pick up the target protein when it was bound to an investigational anti-IP-10 mAb. By replacing the capturing agent by an antibody that recognizes another part of the IP-10 molecule, IP-10 was captured both in its free form and when bound to the mAb, thus providing the total concentration [69]. A distinction between the free and ADA-bound forms of the therapeutic enzyme α-glucosidase was made by capturing the entire IgG fraction of human plasma, including ADAs binding the protein analyte, on a protein G resin and removing the free fraction by washing steps [70]. After elution, the bound fraction was determined by digestion and quantification of a surrogate peptide.

Biotransformation

Despite the thorough characterization of therapeutic proteins in pharmaceutical formulations to guarantee their quality before dosing to patients, there has been relatively little attention for modifications occurring *in vivo*, and their impact on drug safety and efficacy after dosing. Still, biotransformation of proteins is known to take place by reactions such as oxidation, deamidation and isomerization of amino acids and truncation of peptide chains, next to release of the small-molecule drugs (payloads) from ADCs and hydrolysis of the PEG-protein bonds in PEGylated products [71,72]. These structural modifications may be difficult to pick up. Deamidation, for example, leads to a mass increase of just one atomic mass unit, while isomerization only results in a 3D rearrangement of the protein structure. Deamidation of an asparagine in the binding region of trastuzumab, with potential implications for its activity, was found by pellet digestion of patient plasma and quantification of five surrogate peptides that were chromatographically separated: one containing the undeamidated asparagine, three containing the formed aspartate, iso-aspartate and a succinimide intermediate, and one reference peptide from a stable part of the protein. All peptides were found in patient samples, thereby demonstrating extensive biotransformation [73]. Similarly, asparagine deamidation and aspartate isomerization were found in two investigational mAbs, which were isolated from monkey serum by an antihuman IgG antibody [74]. Affinity capture of biotransformation products presents a risk, because the structural changes in the protein may lead to a reduced or even lost recognition by the capturing reagents and, thus, to considerable underestimation of the analyte concentration. Enrichment of trastuzumab from human serum by two different capture formats, based on the pharmacological target, showed substantial differences in the recovered analyte concentrations and this was speculated to be due to biotransformation in the binding region of the analyte [75].

The biotransformation of different parts of a protein can be followed by monitoring multiple surrogate peptides. Dulaglutide, a fusion protein of GLP-1 linked to the Fc domain of IgG4, was extracted from mouse plasma by an antihuman Fc antibody and digested. Two peptides were quantified, one (N-terminal) peptide from the GLP-1 part of the drug and the other from the Fc part. While the concentrations of the Fc part remained high after dosing, the concentrations found for the GLP-1 part decreased and correlated with the concentrations found for the intact protein, as determined with LC–HRMS. Altogether, this suggests biotransformation, and probably proteolytical cleavage, in the N-terminal part of the drug [76]. A similar approach was followed to study the *in vivo* fate of a protein–drug conjugate (PDC): a surrogate peptide from the unconjugated part of the protein was measured next to a peptide containing a small-molecule drug attached via a linker. After enrichment using immobilized metal affinity chromatography, the sample was digested and the two peptides were quantified as a measure for the total and conjugated forms of the PDC, respectively [77]. Biotransformation has also been investigated at the intact

protein level by affinity capture and quantification of a fusion protein and two catabolites by LC–HRMS with deconvolution. Rapid formation of a truncated form of the dosed protein and further truncation were observed after administration to a rabbit [78].

The *in vivo* fate of an ADC is particularly complex, because of the release of the small-molecule payload, including or excluding its linker, and modifications to the intact ADC at the protein and/or payload structure. Therefore, a combination of techniques is required for elucidating the biotransformation of ADCs, such as analysis at the intact protein, subdomain and peptide level as well as quantification of the small-molecule drug and its possible metabolites [79,80]. The concentration of conjugated payload, in other words, all drug molecules that are still bound to an ADC after administration, can be determined by immunocapture with a payload-directed antibody, followed by release of the payload, for example, by a lysosomal enzyme, and its quantification by LC–MS/MS [81,82]. Total antibody levels have been quantified by digestion and measurement of a surrogate peptide after immunocapture with an anti-idiotypic antibody, which captures all ADCs independent of the number of drug molecules. The same approach but using immunocapture with an antipayload antibody, gives the levels of conjugated antibody, in other words, all ADCs with at least one drug [83]. More detailed information about the (remaining) number of drug molecules on an ADC can be obtained by HRMS. This so-called drug–antibody ratio (DAR) has been assessed by immunocapture of the ADC with an anti-idiotypic antibody and subsequent determination of the molecular masses of all different ADC species, from which the different DARs in a sample can be derived [84,85]. The kinetics of *in vivo* drug release from an ADC can depend on the way the drug is linked to the antibody. Two different ADCs with four drug molecules per antibody were studied in monkey plasma by capture with an antihuman IgG antibody and disulfide reduction, followed by LC–Q-TOF-MS analysis of the formed light (25-kDa) and heavy (50-kDa) chains. The original DAR of four remained constant during 3 weeks for an ADC with drug attached to the protein via engineered glutamine linkers, whereas it decreased to three over the same period for an ADC containing engineered cysteine linkers [86]. By including IdeS digestion, an ADC is reduced to fragments of around 25 kDa, from which the mass, and subsequently the DAR, can also be determined [87].

Isoforms

Like biopharmaceuticals, endogenous proteins very often are not single, well-defined species, but exist in multiple, structurally different forms, called isoforms or proteoforms. Depending on the clinical question to be answered, the concentrations of one, several or all of these may be relevant. It is, therefore, important to select an appropriate bioanalytical technique that responds to all isoforms of interest. In a review paper, published in 2016, the relevance of this was indicated for a number of biomarkers [88]. The (intact) 68-amino acid protein RANTES was quantified in human plasma after immunocapture and, next to the full-length protein, several C-terminally and N-terminally truncated forms were found with potential relevance for a patient's state of health. Likewise, the levels of two truncated forms of retinol-binding protein were quantified and found to be elevated in patients with renal failure, and varying concentrations of up to nine isoforms of serum amyloid A were identified in plasma from healthy subjects and patients with Type 2 diabetes or chronic kidney disease. A proper design of the analytical method is very important in this context. Often, only one (recombinant) form of the protein is available as reference standard. If an immunocapture step is included in the workflow, the specificity of the reagent determines if all isoforms are extracted from the biological sample to the same extent. If this is not the case, incorrect concentrations of some of the isoforms would be obtained when quantified against the single recombinant standard. In addition, digestion of the protein isoforms and quantification of surrogate peptides may be a risk, in that some isoforms may only yield useful peptides that are structurally similar, so that they can no longer be separately determined. These issues were addressed in an LC–HRMS method for four isoforms of the biological toxin abrin in human plasma and other complex matrices [89]. An immunocapture step with a mixture of antibodies directed against the different isoforms was employed, in combination with quantification of 14 different surrogate peptides, some common and some isoform-specific. Because of the lack of protein standards, calibrators were used containing the surrogate peptides.

Method validation

Current regulatory guidance documents are still mainly based on the traditional bioanalytical landscape with protein quantification being performed by LBA and small-molecule quantification by LC–MS. They are not completely applicable to newer approaches that include digestion, and hybrid techniques such as immunocapture followed by LC–MS. Now that protein bioanalysis by LC–MS is entering the regulatory field, finetuning of bioanalytical best practices becomes more and more important. Validation experiments have been suggested to address new parameters

like immunocapture efficiency and digestion efficiency, and alternative approaches for assessing matrix effect and selectivity/specificity [90]. When it comes to acceptance of results, there is ongoing debate about which criteria to apply: those traditionally used for LC–MS or those traditionally used for proteins (by LBA). Recommendations of industry experts have been published and current thinking is that, because of the complexity of the developing science of protein bioanalysis by LC–MS, there is no compelling reason to raise the standard over the currently accepted criteria for LBA [91,92].

Conclusion

Since the start of this journal 10 years ago, LC–MS has emerged as a quantitative tool for protein bioanalysis, as an alternative for or an addition to the traditional LBA-based approaches. Next to its reliable quantitative performance, the main advantage of LC–MS is related to its ability to provide structural information about a protein, or at least part of a protein, which is inherently impossible by LBA. A weaker point is its often limited detection sensitivity, but much technical progress has been made over the last decade. For many applications, sufficient sensitivity can now be obtained, in particular when LC–MS is used in combination with an (immuno)capture step. LC–MS is especially attractive for preclinical drug development, where concentrations are relatively high and the structures of the human or humanized protein drugs of interest allow a relatively simple extraction from and detection in animal matrices. In human samples, the use of LC–MS is more complicated because of the close structural similarity of analyte and endogenous matrix proteins. A risk of protein LC–MS and LBA alike, is the fact that the required method sensitivity and selectivity often dictate the choice for a particular technical approach, which may not always provide concentration results that are meaningful. The occurrence of protein isoforms and biotransformation, as well as the binding to one or more endogenous macromolecules, result in a variety of protein forms in a sample that may, may not or may only partly respond to the selected bioanalytical technique. Protein concentration results should, therefore, always be evaluated by referring to the experimental approach that was followed and the materials that were used.

Future perspective

LC–MS for quantitative protein bioanalysis is here to stay. In the near future, it will not replace but increasingly complement LBAs in drug development and patient care, because it adds value in our understanding of the *in vivo* fate of dosed biopharmaceuticals and endogenous biomarkers. From a technical point of view, it is to be expected that more developments will occur in the field of intact protein analysis. HRMS is increasingly being introduced in laboratories for quantitative bioanalysis and with these instruments becoming more sensitive, robust and affordable, more scientists will likely try to move from digestion of a protein and indirect quantification via a surrogate peptide to direct analysis of the intact analyte. In this respect, technical advances are needed when it comes to stationary phases for LC and it is hoped that the increased interest in intact protein analysis will stimulate the development of materials with improved separation properties for protein forms. Similarly, user-friendly software solutions for robust deconvolution of the highly complex mass spectra, in compliance with regulatory expectations, are desirable. Not less importantly, as our knowledge of protein bioanalysis increases and more and more technical possibilities become available, it can be anticipated that there will be more attention for selection of the best, or rather most appropriate, method. The usefulness of concentration results will improve if the bioanalytical approach is based on the specific scientific question to be answered, rather than using a technique that happens to be around. Finally, protein bioanalysis is a truly interdisciplinary science and much of its success is due to the collaboration of researchers with very different backgrounds. It is our hope and expectation that the bioanalytical community will continue to work together to help advance our knowledge in this fascinating field.

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Executive summary

- LC–MS has become a well-established bioanalytical platform for protein quantification.
- Over the past decade, developments have focused on enhancing the technical possibilities of protein LC–MS and on improving our understanding of what a concentration result means.
- Very often, protein quantification is based on digestion of the analyte and subsequently measuring one or more surrogate peptides, but methods for intact proteins are increasingly being used.
- By properly combining extraction, digestion, separation and detection approaches, protein concentrations down to the pg/ml level can be reliably determined.
- Conceptually, protein quantification usually is not straightforward because of the potential occurrence of many different protein forms in a sample.
- Since the technical approach that is used typically determines which protein form is quantified, it is important to have a good understanding of both the bioanalytical question and the bioanalytical method.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Campbell JL, Le Blanc JCY. Peptide and protein drug analysis by MS: challenges and opportunities for the discovery environment. *Bioanalysis* 3(6), 645–657 (2011).
2. Ewles M, Goodwin L. Bioanalytical approaches to analyzing peptides and proteins by LC–MS/MS. *Bioanalysis* 3(12), 1379–1397 (2011).
3. Li F, Fast D, Michael S. Absolute quantitation of protein therapeutics in biological matrices by enzymatic digestion and LC–MS. *Bioanalysis* 3(21), 2459–2480 (2011).
4. Szapacs M, Mencken T, Williams J, Li Y. Utilizing digestion procedures in the bioanalytical laboratory. *Bioanalysis* 8(1), 29–36 (2016).
5. Zhang G, Sexton DJ, Faucette RR *et al.* 2D-LC–MS/MS to measure cleaved high-molecular weight kininogen in human plasma as a biomarker for C1-INH-HAE. *Bioanalysis* 9(19), 1477–1491 (2017).
6. Hansen K, Szarka S, Escoffier E *et al.* Glu-C, an alternative digestive enzyme for the quantitative LC–MS/MS analysis of an IgG-based antibody biotherapeutic. *Bioanalysis* 10(13), 997–1007 (2018).
7. Fung EN, Zambito F, Haulenbeek J *et al.* Targeting an acid labile aspartyl-prolyl amide bond as a viable alternative to trypsin digestion to generate a surrogate peptide for LC–MS/MS analysis. *Bioanalysis* 6(22), 2985–2998 (2014).
8. Ouyang Z, Furlong MT, Wu S *et al.* Pellet digestion: a simple and efficient sample preparation technique for LC–MS/MS quantification of large therapeutic proteins in plasma. *Bioanalysis* 4(10), 17–28 (2012).

• **First report about the advantages of pellet digestion.**

9. Yuan L, Arnold ME, Aubry A-F, Ji QC. Simple and efficient digestion of a monoclonal antibody in serum using pellet digestion: comparison with traditional digestion methods in LC–MS/MS bioanalysis. *Bioanalysis* 4(24), 2887–2896 (2012).
10. Shida H, Naito T, Shibata K *et al.* LC–MS/MS method for denosumab quantitation in human serum with rapid protein digestion using immobilized trypsin. *Bioanalysis* 10(18), 1501–1510 (2018).
11. Regnier FE, Kim J. Accelerating trypsin digestion: the immobilized enzyme reactor. *Bioanalysis* 6(19), 2685–2698 (2014).
12. Iwamoto N, Takanashi M, Umino Y *et al.* Application of nano-surface and molecular-orientation limited proteolysis to LC–MS bioanalysis of cetuximab. *Bioanalysis* 8(10), 1009–1020 (2016).
13. Gong C, Zeng J, Akinsanya B *et al.* Development and validation of an LC–MS/MS assay for the quantitation of a PEGylated anti CD28 domain antibody in human serum: overcoming interference from antidrug antibodies and soluble target. *Bioanalysis* 6(18), 2371–2383 (2014).
14. Bronsema KJ, Bischoff R, van de Merbel NC. High-sensitivity LC–MS/MS quantification of peptides and proteins in complex biological samples: the impact of enzymatic digestion and internal standard selection on method performance. *Anal. Chem.* 85(20), 9528–9535 (2013).
15. Wright K, Dufield D. Minimalistic sample preparation strategies for LC–MS quantification of large molecule biopharmaceuticals: a case study highlighting alpha-1 antitrypsin protein. *Bioanalysis* 6(13), 1813–1825 (2014).
16. Pu J, An B, Vazvaei F, Qu J. Bioanalysis. Enrichment of protein therapeutics and biomarkers for LC–MS quantification. *Bioanalysis* 10(13), 979–982 (2018).
17. Wilffert D, Bischoff R, van de Merbel NC. Antibody-free workflows for protein quantification by LC–MS/MS. *Bioanalysis* 7(6), 763–779 (2015).

• **Comprehensive overview of generic protein-enrichment possibilities.**

18. Wilffert D, Asselman A, Donzelli R *et al.* Highly sensitive antibody-free μLC–MS/MS quantification of rhTRAIL in serum. *Bioanalysis* 8(9), 881–890 (2016).

19. Bronsema KJ, Bischoff R, Bouche M-P *et al.* High-sensitivity quantitation of a Nanobody® in plasma by single-cartridge multidimensional SPE and ultra-performance LC–MS/MS. *Bioanalysis* 7(1), 53–64 (2015).
20. Wilffert D, Donzelli R, Asselman A *et al.* Quantitative antibody-free LC–MS/MS analysis of sTRAIL in sputum and saliva at the sub ng/mL level. *J. Chromatogr. B* 1032, 205–210 (2016).
21. Shi J, Wong JM, Ma J *et al.* Reagent-free LC–MS/MS-based pharmacokinetic quantification of polyhistidine-tagged therapeutic proteins. *Bioanalysis* 9(3), 251–264 (2017).
22. Fung EN, Bryan P, Kozhich A. Techniques for quantitative LC–MS/MS analysis of protein therapeutics: advances in enzyme digestion and immunocapture. *Bioanalysis* 8(8), 847–856 (2016).
23. Zhao Y, Gu H, Zheng N, Zeng J. Critical considerations for immunocapture enrichment LC–MS bioanalysis of protein therapeutics and biomarkers. *Bioanalysis* 10(13), 987–995 (2018).
24. Vialaret J, Broutin S, Pugnier C *et al.* What sample preparation should be chosen for targeted MS monoclonal antibody quantification in human serum? *Bioanalysis* 10(10), 723–735 (2018).
25. Sleczka BG, Mehl JT, Shuster DJ *et al.* Quantification of human mAbs in mouse tissues using generic affinity enrichment procedures and LC–MS detection. *Bioanalysis* 6(13), 1795–1811 (2014).
26. Lee JW. Generic method approaches for monoclonal antibody therapeutics analysis using both ligand binding and LC–MS/MS techniques. *Bioanalysis* 8(1), 19–27 (2016).
27. Chen L, Liu S, Pagels S *et al.* Development of an ELISA-LC–MS hybrid assay for quantification of biotherapeutics. *Bioanalysis* 10(17), 1427–1438 (2018).
28. Wang Q, Han J, Sha C *et al.* Novel strategy using tryptic peptide immunoaffinity-based LC–MS/MS to quantify denosumab in monkey serum. *Bioanalysis* 9(19), 1451–1463 (2017).
29. Gupta V, Lassman ME, Lee AYH *et al.* An evaluation of an aptamer for use as an affinity ligand reagent with MS: PCSK9 as an example protein. *Bioanalysis* 8(15), 1557–1564 (2016).
30. Klont F, Hadderingh M, Horvatovich P *et al.* Affimers as an alternative to antibodies in an affinity LC–MS assay for the quantification of the soluble receptor of advanced glycation end-products (sRAGE) in human serum. *J. Prot. Res.* 17(8), 2892–2899 (2018).
31. Stevenson D, El-Sharif HF, Reddy SM. Selective extraction of proteins and other macromolecules from biological samples using molecular imprinted polymers. *Bioanalysis* 8(21), 2255–2263 (2016).
32. Yang W, Kernstock R, Simmons N, Alak A. ELISA microplate: a viable immunocapture platform over magnetic beads for immunoaffinity-LC–MS/MS quantitation of protein therapeutics? *Bioanalysis* 7(3), 307–318 (2015).
33. Lanshoeft C, Heudi O, Cianferani S *et al.* Quantitative analysis of hIgG1 in monkey serum by LC–MS/MS using mass spectrometric immunoassay. *Bioanalysis* 8(10), 1035–1049 (2016).
34. Becker JO, Hoofnagle AN. Replacing immunoassays with tryptic digestion-peptide immunoaffinity enrichment and LC–MS/MS. *Bioanalysis* 4(3), 281–290 (2012).
35. Sucharski FK, Meier S, Miess C *et al.* Development of an automated, interference-free, 2D-LC–MS/MS assay for quantification of a therapeutic mAb in human sera. *Bioanalysis* 10(13), 1023–1037 (2018).
36. Schultz GA, McCardle K, Neubert H. Large-scale implementation of sequential protein and peptide immunoaffinity enrichment LC/nanoLC–MS/MS for human β-nerve growth factor. *Bioanalysis* 8(8), 753–764 (2016).

•• **Impressive methodology for routine trace-level quantification of a biomarker by pre- and postdigestion immunocapture.**

37. Howard JW, Kay RG, Pleasance S, Creaser CS. UHPLC for the separation of proteins and peptides. *Bioanalysis* 4(24), 2971–2988 (2012).
38. Kleinnijenhuis AJ, Ingola M, Toersche JH *et al.* Quantitative bottom up analysis of infliximab in serum using protein A purification and integrated μLC-electrospray chip IonKey MS/MS technology. *Bioanalysis* 8(9), 891–904 (2016).
39. Lassman ME, Fernandez-Metzler C. Applications of low-flow LC-SRM for the analysis of large molecules in pharmaceutical R&D. *Bioanalysis* 6(13), 1859–1867 (2014).
40. Shi T, Qian W-J. Antibody-free PRISM-SRM for multiplexed protein quantification: is this the new competition for immunoassays in bioanalysis? *Bioanalysis* 5(3), 267–269 (2013).
41. Sandra K, Mortier K, Jorge L *et al.* LC–MS/MS quantification of next-generation biotherapeutics: a case study for an IgE binding Nanobody in cynomolgus monkey plasma. *Bioanalysis* 6(9), 1201–1213 (2014).
42. Sandra K, Sandra P. The opportunities of 2D-LC in the analysis of monoclonal antibodies. *Bioanalysis* 7(22), 2843–2847 (2015).
43. Xu K, Liu L, Maia M *et al.* A multiplexed hybrid LC–MS/MS pharmacokinetic assay to measure two co-adminstered monoclonal antibodies in a clinical study. *Bioanalysis* 6(13), 1781–1794 (2014).
44. Zhu Y, D'Arienzo C, Lou Z *et al.* LC–MS/MS multiplexed assay for the quantitation of a therapeutic protein BMS-986089 and the target protein Myostatin. *Bioanalysis* 8(3), 193–204 (2016).
45. Mekhssian K, Mess J-N, Garofolo F. Application of high-resolution MS in the quantification of a therapeutic monoclonal antibody in human plasma. *Bioanalysis* 6(13), 1767–1779 (2014).

46. Plumb RS, Fujimoto G, Mather J *et al.* Comparison of the quantification of a therapeutic protein using nominal and accurate mass MS/MS. *Bioanalysis* 4(5), 605–615 (2012).
47. Bults P, Meints M, Sonesson A *et al.* Improving selectivity and sensitivity of protein quantitation by LC-HR-MS/MS: determination of somatropin in rat plasma. *Bioanalysis* 10(13), 1009–1021 (2018).
48. Kay RG, Hoard J, Stensson S. A current perspective of supercharging reagents and peptide bioanalysis. *Bioanalysis* 8(3), 157–161 (2016).
49. Furlong MT, Zhao S, Mylott W *et al.* Dual universal peptide approach to bioanalysis of human monoclonal antibody protein drug candidates in animal studies. *Bioanalysis* 5(11), 1363–1376 (2013).

•• **Description of a universal approach to quantify human monoclonal antibodies in animal matrices by a single LC–MS method.**

50. Furlong MT, Titsch C, Xu W *et al.* An exploratory universal LC–MS/MS assay for bioanalysis of hinge region-stabilized human IgG4 mAbs in clinical studies. *Bioanalysis* 6(13), 1747–1758 (2014).
51. Kaur S, Liu L, Cortes DF *et al.* Validation of a biotherapeutic immunoaffinity-LC–MS/MS assay in monkey serum: 'plug-and-play' across seven molecules. *Bioanalysis* 8(15), 1565–1577 (2016).
52. Van den Broek I, van Dongen WD. LC–MS-based quantification of intact proteins: perspective for clinical and bioanalytical applications. *Bioanalysis* 7(15), 1943–1958 (2015).
53. Lanshoeft C, Cianferani S, Heudi O. Generic hybrid ligand binding assay liquid chromatography high-resolution mass spectrometry-based workflow for multiplexed human immunoglobulin G1 quantification at the intact protein level: application to preclinical pharmacokinetic studies. *Anal. Chem.* 89(4), 2628–2635 (2017).
54. Ramagiri S, Garofolo F. Large molecule bioanalysis using Q-TOF without predigestion and its data processing challenges. *Bioanalysis* 4(5), 529–540 (2012).
55. Jian W, Kang L, Burton L, Weng N. A workflow for absolute quantitation of large therapeutic proteins in biological samples at intact level using LC-HRMS. *Bioanalysis* 8(16), 1679–1691 (2016).

•• **First report of the possibility to quantify an intact monoclonal antibody by LC–MS.**

56. Qiu X, Kang L, Case M *et al.* Quantitation of intact monoclonal antibody in biological samples: comparison of different data processing strategies. *Bioanalysis* 10(13), 1055–1067 (2018).
57. Jin W, Burton L, Moore I. LC-HRMS quantitation of intact antibody drug conjugate trastuzumab emtansine from rat plasma. *Bioanalysis* 10(11), 851–862 (2018).
58. Kellie JF, Kehler JR, Karlinsey MZ, Summerfield SG. Toward best practices in data processing and analysis for intact biotherapeutics by MS in quantitative bioanalysis. *Bioanalysis* 9(23), 1883–1893 (2017).
59. Zhang L, Vasicek LA, Hsieh SC *et al.* Top-down LC–MS quantitation of intact denatured and native monoclonal antibodies in biological samples. *Bioanalysis* 10(13), 1039–1054 (2018).

• **Insightful report on the possibility for quantitative bioanalysis of large proteins in their native form.**

60. Kellie JF, Kehler JR, Mencken T *et al.* A whole-molecule immunocapture LC–MS approach for the *in vivo* quantitation of biotherapeutics. *Bioanalysis* 8(20), 2103–2114 (2016).
61. Van de Merbel NC, Bronsema KJ, Nemansky M. Protein quantification using LC–MS: can it make a difference? *Bioanalysis* 4(17), 2113–2116 (2012).
62. Heinrich J, Staack RF, Stubenrauch K-G, Papadimitriou A. Proposal for a harmonized descriptive analyte nomenclature for quantitative large-molecule bioanalysis. *Bioanalysis* 7(24), 3057–3062 (2015).
63. Zhang YJ, Olah TV, Zeng J. The integration of ligand binding and LC–MS-based assays into bioanalytical strategies for protein analysis. *Bioanalysis* 6(13), 1827–1841 (2014).
64. Zhang YJ, Luo L, Desai DD. Overview on biotherapeutic proteins: impact on bioanalysis. *Bioanalysis* 8(1), 1–9 (2016).
65. White JT, Bonilla LE. Free and total biotherapeutic evaluation in chromatographic assays: interference from targets and immunogenicity. *Bioanalysis* 4(19), 2401–2411 (2012).

•• **Excellent overview of the analytical consequences of protein–protein interactions.**

66. Staack RF, Jordan G, Dahl G, Heinrich J. Free analyte concept: a novel approach to prove correct quantification of free therapeutic protein drug/biomarker concentrations. *Bioanalysis* 6(4), 485–496 (2014).
67. Jordan G, Onami I, Heinrich J, Staack RF. Evaluation of the potential use of hybrid LC–MS/MS for active drug quantification applying the 'free analyte QC concept'. *Bioanalysis* 9(21), 1705–1717 (2017).

•• **Convincing description of the possibilities to establish the free concentration of a protein by LC–MS.**

68. Ewles M, Mannu R, Fox C *et al.* LC–MS/MS strategies for therapeutic antibodies and investigation into the quantitative impact of anti-drug antibodies. *Bioanalysis* 8(24), 2565–2579 (2016).
69. Zhang H, Gu H, Shipkova P *et al.* Immunoaffinity LC–MS/MS for quantitative determination of a free and total protein target as a target engagement biomarker. *Bioanalysis* 9(20), 1573–1588 (2017).

70. Bronsema KJ, Bischoff R, Pijnappel WWMP *et al.* Absolute quantification of the total and antidrug antibody-bound concentrations of recombinant human α-glucosidase in human plasma using protein G extraction and LC–MS/MS. *Anal. Chem.* 87(8), 4394–4401 (2015).

71. Hamuro LL, Kishnani NS. Metabolism of biologics: biotherapeutic proteins. *Bioanalysis* 4(2), 189–195 (2012).

72. Kellie JF, Karlinsey MZ. Review of approaches and examples for monitoring biotransformation in protein and peptide therapeutics by MS. *Bioanalysis* 10(22), 1877–1890 (2018).

- **Good overview of protein biotransformation and its bioanalytical consequences.**

73. Bults P, Bischoff R, Bakker H *et al.* LC–MS/MS-based monitoring of *in vivo* protein biotransformation: quantitative determination of tratstuzumab and its deamidation products in human plasma. *Anal. Chem.* 88(3), 1871–1877 (2016).

74. Mehl JT, Sleczka BG, Ciccimaro F *et al.* Quantification of *in vivo* site-specific Asp isomerization and Asn deamidation of mAbs in animal serum using IP-LC–MS. *Bioanalysis* 8(15), 1611–1622 (2016).

75. Liu L, Xu K, Li J *et al.* Optimizing hybrid LC–MS/MS binding conditions is critical: impact of biotransformation on quantification of trastuzumab. *Bioanalysis* 10(22), 1819–1831 (2018).

76. Kang L, Camacho RC, Li W *et al.* Simultaneous catabolite identification and quantitation of large therapeutic protein at the intact level by immunoaffinity capture liquid chromatography–high-resolution mass spectrometry. *Anal. Chem.* 89(11), 6065–6075 (2017).

77. Shi C, Goldberg S, Lin T *et al.* Bioanalytical workflow for novel scaffold protein-drug conjugates: quantitation of total centyrin protein, conjugated centyrin and free payload for centyrin-drug conjugate in plasma and tissue samples using liquid chromatography–tandem mass spectrometry. *Bioanalysis* 10(20), 1651–1665 (2018).

78. Zell M, Husser C, Staack RF *et al. In vivo* biotransformation of the fusion protein tetranectin-apolipoprotein A1 analyzed by ligand-binding mass spectrometry combined with quantitation by ELISA. *Anal. Chem.* 88(23), 11670–11677 (2016).

79. Saad OM, Shen B-Q, Xu K *et al.* Bioanalytical approaches for characterizing catabolism of antibody-drug conjugates. *Bioanalysis* 7(13), 1583–1604 (2015).

- **Perspective on the bioanalysis of antibody–drug conjugates and their biotransformation products.**

80. Tumey LN, Rago B, Han X. *In vivo* biotransformation of antibody-drug conjugates. *Bioanalysis* 7(13), 1649–1664 (2015).

81. Myler H, Rangan VS, Wang J *et al.* An integrated multiplatform bioanalytical strategy for antibody-drug conjugates: a novel case study. *Bioanalysis* 7(13), 1569–1582 (2015).

82. Sanderson RJ, Nicholas ND, Baker Lee C *et al.* Antibody-conjugated drug assay for protease-cleavable antibody-drug conjugates. *Bioanalysis* 8(1), 55–63 (2016).

83. Wang J, Gu H, Liu A *et al.* Antibody-drug conjugate bioanalysis using LB-LC–MS/MS hybrid assays: strategies, methodology and correlation to ligand-binding assays. *Bioanalysis* 8(13), 1383–1401 (2016).

84. Kaur S, Xu K, Saad OM *et al.* Bioanalytical assay strategies for the development of antibody-drug conjugate biotherapeutics. *Bioanalysis* 5(2), 201–226 (2013).

85. Xu K, Liu L, Dere R *et al.* Characterization of the drug-to-antibody ratio distribution for antibody-drug conjugates in plasma/serum. *Bioanalysis* 5(9), 1057–1071 (2013).

86. Grafmuller L, Wei C, Ramanathan R *et al.* Unconjugated payload quantification and DAR characterization of antibody-drug conjugates using high-resolution MS. *Bioanalysis* 8(16), 1663–1678 (2016).

87. Davis JA, Kagan M, Read J *et al.* Immunoprecipitation middle-up LC–MS for *in vivo* drug-to-antibody ratio determination for antibody-drug conjugates. *Bioanalysis* 9(20), 1535–1549 (2017).

88. Trenchevska O, Nelson RW, Nedelkov D. Mass spectrometric immunoassays for discovery, screening and quantification of clinically relevant proteoforms. *Bioanalysis* 8(15), 1623–1633 (2016).

89. Hansbauer E-M, Worbs S, Volland H *et al.* Rapid detection of abrin toxin and its isoforms in complex matrices by immuno-extraction and quantitative high resolution targeted mass spectrometry. *Anal. Chem.* 89(21), 11719–11727 (2017).

90. Duggan J, Ren B, Mao Y *et al.* LC–MS quantification of protein drugs: validating protein LC–MS methods with predigestion immunocapture. *Bioanalysis* 8(18), 1951–1964 (2016).

91. Knutsson M, Schmidt R, Timmerman P. LC–MS/MS of large molecules in a regulated environment – which acceptance criteria to apply? *Bioanalysis* 5(18), 2211–2214 (2013).

92. Duggan JX, Vazvaei F, Jenkins R. Bioanalytical method validation considerations for LC–MS/MS assays of therapeutic proteins. *Bioanalysis* 7(11), 1389–1395 (2015).

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Bioanalysis

Development, validation and application of a liquid chromatography–tandem mass spectrometry method for the activity and inhibition of DPP-4

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Background: Inhibition of the enzyme dipeptidyl peptidase 4 (DPP-4) is a pharmaceutical treatment for type 2 diabetes. To demonstrate bioequivalence of enzyme inhibition of a new dosage form of the inhibitor vildagliptin, a method for enzyme activity was developed, validated and applied using liquid chromatography and tandem mass spectrometry (LC-MS/MS). **Results:** The method was validated fit for purpose, including accuracy, precision as well as the stability of the activity and the inhibition of DPP-4 in human plasma. **Conclusion:** A method for the determination of the activity and inhibition of DPP-4 was developed using LC-MS/MS readout; the characteristics and performance of the method met predefined acceptance criteria and were fit for the purpose of a bioequivalence clinical trial.

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Keywords: accuracy • bioequivalence • DPP-4 • enzyme inhibition • LC-MS/MS • validation • vildagliptin

Dipeptidyl peptidase-4 (DPP-4) cleaves the dipeptides X-proline and X-alanine from the N-terminus of a broad range of endogenous polypeptide substrates and thus plays an important role in the regulation of polypeptide hormones and enzymes in plasma. This includes several key proteins involved in the regulation of blood glucose levels such as GLP-1 and GIP, which become inactivated by DPP-4 but stay upregulated when DPP-4 is inhibited, thereby prolonging their activity to decrease blood glucose levels after food intake [1,2].

The first approved drug in the class of DPP-4 inhibitors was sitagliptin in 2006. Today more than 10 drugs are available in this class, called gliptins, that result in inhibition of DPP-4. These gliptins differ in their chemical structure, bind different epitopes of the DPP-4 enzyme and hence they have different selectivity and potency for DPP-4 inhibition. Examples of these gliptins include vildagliptin, saxagliptin and linagliptin. The pharmacokinetics of the gliptin drug itself, as well as the pharmacodynamic effect of the inhibitor on the activity of DPP-4, has been of interest from the onset of the gliptin class developments [3]. Since then, different methods have been developed to measure DPP-4 activity and inhibition in clinical study samples; some of those, such as colorimetric kits; substrates for the development of new enzymatic assays can also be purchased commercially.

Activity measurements are commonly performed using colorimetric detection. Ninety-six-well plate readers can monitor conversion reaction kinetics in individual wells, representing the enzyme activity in the corresponding sample. A liquid chromatography and tandem mass spectrometry (LC-MS/MS) method using protein precipitation, ultra HPLC (UHPLC) chromatographic separation and highly selective mass spectrometric detection in multiple reaction monitoring (MRM) mode, is expected to be less affected by interferences from the matrix than such colorimetric assays. Another recent method used UHPLC with fluorescence detection, which already constitutes a significant improvement in selectivity [4]. However, mass spectrometric detection combined with the use of a stable labeled isotope internal standard has become the preferred approach in bioanalysis of small molecules. On the other hand, the colorimetric approach has the advantages of lower cost for equipment, using fewer chemicals and being

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considerably more rapid, as the measurement is commonly performed in 1–5 min per 96-well plate whereas the presented LC-MS/MS method requires almost 5 h of analysis time. Considering the importance of the results for the outcome of the bioequivalence trial, the Indian Health authorities requested the use of LC-MS/MS for this project.

In this work, we describe the development and validation of the first analytical LC-MS/MS method for determination of the activity and inhibition of DPP-4 to show bioequivalence of a modified release formulation of 100 mg vildagliptin once daily with the established 50-mg immediate release formulation twice daily. The primary objective of the trial was defined as the bioequivalence of the inhibition of DPP-4 activity.

Experimental

Chemicals & reagents

Gly-Pro para-nitroaniline (GP-p-NA) was purchased from Bachem AG (Bubendorf, Germany); para-nitroanilide (p-NA), vildagliptin, trichloroacetic acid (TCA), 2-propanol, methanol, acetonitrile, Dulbecco's phosphate buffered saline (PBS) and formic acid were ordered from Merck KGaA (MO, USA); D_4-p-NA was ordered from Cambridge Isotope Laboratories (MA, USA).

Preparation of stock, calibration & quality control solutions

Two separate stock solutions of p-NA were prepared by dissolving an exact weighed amount of methanol, correcting for purity and salt form to a concentration of 10.0 mM. One stock solution was used to prepare calibration pools at 1.00, 2.00, 5.00, 25.0, 100, 250, 400 and 500 µM and from the other solution, validation and quality control (QC) samples were prepared at 1.00, 3.00, 200, 375 and 500 µM. Intermediate solutions were prepared in methanol as needed. A stock solution for vildagliptin was prepared at 1.00 mg/ml by dissolving a weighed amount in water, correcting for purity and salt form. Plasma samples for use as activity QC or activity stability assessment were divided into a sufficient number of aliquots and stored at -20°C. QC samples were prepared neat (uninhibited), after partial inhibition by adding 5.00 ng/ml or after complete inhibition by adding 500 ng/ml vildagliptin, which correspond to no inhibition, low inhibition and high inhibition, respectively. A stock solution of D_4-p-NA was prepared in the same manner as for p-NA. A working solution of this internal standard was made at 50 µM in acetonitrile. p-NA and D_4-p-NA stock solutions were prepared in polypropylene tubes and stored at -20°C.

Enzymatic conversion & sample extraction

Aliquots of 50.0-µl plasma samples were pipetted into a 1-ml polypropylene 96-well plate and equilibrated to the reaction temperature of 20°C in a thermomixer (Eppendorf, Hamburg, Germany) equipped with a heated cover for 10 min. Twenty-five microliters of freshly prepared substrate solution (839 µM GP-p-NA dissolved in PBS, final concentration in the reaction is 280 µM) was added at ambient temperature, and a timer was used to stop the reaction after exactly 15.0 min. The reaction was stopped by the addition of 50.0 µl of 10% TCA and 100 µl of acetonitrile to the sample. Twenty-five microliters of the internal standard working solution was also added to the samples, followed by mixing for 10 s at 1600 rpm and centrifugation for 5 min at 3500× *g*. From the supernatant solution, 50 µl was transferred to a clean plate using an automated liquid handling system (Tomtec Inc., CT, USA) and diluted with 200 µl of ultrapure water. After mixing, the samples were stored in the autosampler at 4°C until 5 µl of the extract was injected for LC-MS/MS analysis.

For the plasma samples spiked with p-NA, calibration curve, validation and QC samples, PBS was added without substrate. For other samples, the same procedure was applied.

LC-MS/MS method

The product of the reaction between DPP-4 and its substrate GP-p-NA was chromatographically separated from interferences using an Acquity UPLC BEH C18 50 × 2.1 mm 1.7-µm analytical column (Waters Corporation, MA, USA) at a temperature of 40°C using a flow of 1 ml/min with an 1290 Infinity II UHPLC system (Agilent, CA, USA). The mobile phases were A – 0.1% formic acid in water and B – acetonitrile. The method uses isocratic elution with 15% B for 1.1 min, after which the column is first washed at 50% B for 1.6 min, followed by a second washout step at 95% B from 1.7 to 2.4 min followed by reequilibration at initial conditions to prepare for the subsequent injection. The total runtime was 3 min, of which only the time between 0.4 and 1.0 min were recorded by scheduled ionization to prevent fouling of the ionization source. p-NA and D_4-p-NA eluted with a retention time of 0.7 min.

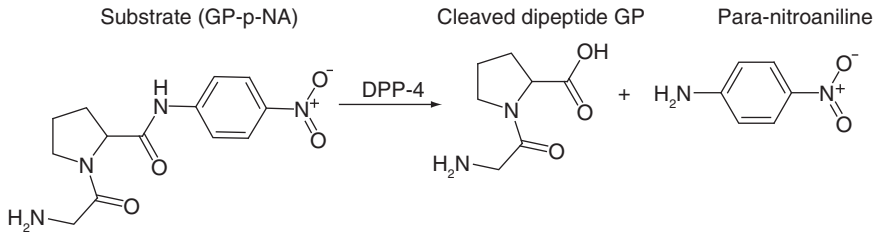

Figure 1. Conversion of the substrate Gly-Pro para-nitroaniline (GP-p-NA) to GP and p-NA by DPP-4.

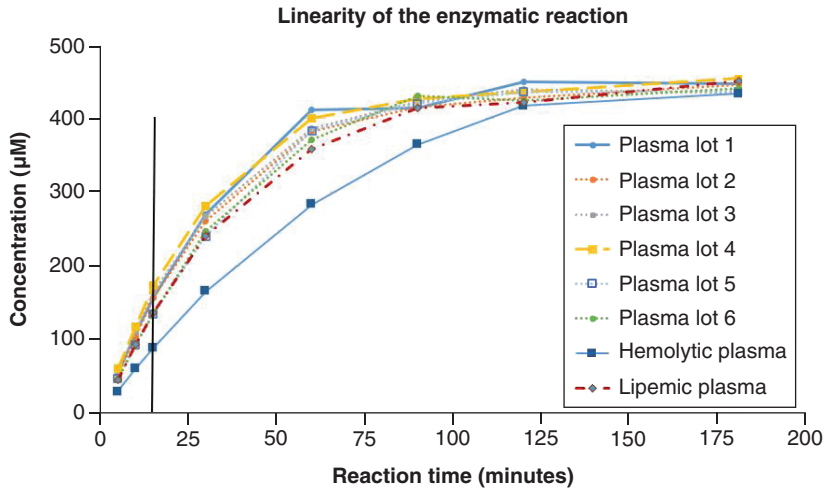

Figure 2. Time course of the substrate conversion by DPP-4. The 15-min time point proceeds the onset of nonlinearity from depletion of the substrate.

The triple quad API-4000 MS detector (AB SCIEX, MA, USA) was used in positive ion mode at unit resolution and monitored the transitions of 139.1–76.0 m/z and 143.1–80.0 m/z for p-NA and D_4-p-NA respectively, with a dwell time of 100 ms each. The ionization conditions were optimized for signal to noise. The method used a temperature of 500°C and a ionspray voltage of 5500 V. The fragments were formed by collision with nitrogen gas with a collision energy of 42 V.

Results & discussion

Linearity of the substrate conversion reaction

The speed of the enzymatic conversion is determined by several factors. Initially, the substrate GP-p-NA is available in excess and the speed of the reaction is determined solely by the activity of DPP-4 in the sample. During this phase, the reaction (shown in Figure 1) follows zero-order kinetics and thus proceeds in a linear fashion. Once the excess of substrate is depleted, the availability of substrate limits the reaction speed, and the curve starts to flatten. When the substrate is fully depleted, a longer reaction time no longer increases the concentration of p-NA in the sample. So with unlimited reaction time, ultimately all samples will give the same final concentration of p-NA, regardless of the activity of DPP-4. For calculating relative inhibition of DPP-4 in study samples, it is important to stop the reaction and measure the p-NA concentrations formed while the reaction is still on the linear part of the curve. On the basis of the data in Figure 2, we concluded that a reaction time of 15 min under the chosen incubation conditions is within the linear range for all tested plasma lots.

Reaction time

The timing of starting the reaction by adding the GP-p-NA substrate and stopping it by addition of the TCA/acetonitrile solution is critical. The reaction time was set at 15.0 min so that the relative impact of variations in reaction time within a batch of samples was minimal while still being well within the linear phase of product formation.

Table 1. Overview of the validation results.

Item	Result
p-NA spiked in plasma	
Linearity	Correlation of ≥0.9970 between 1.00 and 500 μM
Accuracy	Overall bias ranged from -4.3 to 1.9%
Precision	Overall CV ranged from 1.4 to 6.4%
Selectivity	No interfering peaks in six plasma lots plus one lipemic and one hemolytic lot; the presence of vildagliptin does not interfere
Matrix variability	CV 5.1% and average bias -10.5% for six plasma lots; in hemolytic plasma, bias is less than -7.0%, and in lipemic plasma less than -14%
Matrix effect	Relative matrix factor is 1.03, and CV is 2.5%
Recovery	92.0% or higher, also for the d4 internal standard
Carryover	Below 20.0% of LLOQ in blanks following ULOQ or high QC
Stability of p-NA	
Autosampler	Reinjection of a run does not give significantly different results after 145 h at +4°C
Plasma	24-h benchtop; 204 days frozen storage; five freeze–thaw cycles at -20 and -80°C
Activity of DPP-4	
Accuracy	The biases from the established values were -6.24%, -5.59% and -10.5% at resp. high, low and no inhibition
Precision	The CVs were 16.2%, 10.8% and 8.9% at high, low and no inhibition, respectively
Linearity/selectivity	The reaction was linear for 15 min in six plasma lots, plus one lipemic and one hemolytic plasma lot
Inhibition of DPP-4	
Accuracy	The biases from the established values were -0.17% and -2.85% at high and low inhibition, respectively
Precision	The CVs were +0.19% and +2.57% at high and low inhibition, respectively
Stability of activity and inhibition	
Plasma	23 h both on ice and on the benchtop
	Four freeze–thaw cycles, from -20 and -70°C to benchtop
	204 days storage at -20 and -70°C
Autosampler	97 h of extract storage at +4°C
Whole blood	2 h at ambient conditions and on ice

CV: Coefficient of variation; LLOQ: Lower limit of quantitation; QC: Quality control; ULOQ: Upper limit of quantitation.

Stopping the reaction, residual activity in the autosampler

The current method uses both 10% TCA and acetonitrile to stop the reaction and precipitate out the proteins in the sample. Initially during method development only acetonitrile was used, and we observed residual DPP-4 activity in the supernatant, even after removing the protein pellet from the extract. By adding 10% TCA to the solution, residual activity was significantly reduced, and the autosampler stability test passed within criteria, as shown in Table 1.

Linear concentration range

The analytical calibration range of p-NA concentrations could only be determined after optimizing the enzymatic reaction conditions because these greatly affect the formed amount of p-NA.

An LLOQ of 1.00 μM p-NA allowed for accurate quantification of p-NA levels in plasma samples with up to 99% inhibition. Figure 3 shows the LC-MS/MS chromatograms of a blank plasma (processed without adding substrate), an LLOQ sample from the calibration curve and a chromatogram from the analysis of a plasma sample inhibited with 500 ng/ml of vildagliptin, corresponding to 97% inhibition. The peak area in the inhibited sample is approximately a factor of three higher than the LLOQ sample. The highest value found after 15-min reaction time using an uninhibited sample in the selectivity experiment was ~200 μM p-NA. Using a safety margin to account for potential biological variation, the ULOQ of the method was set to 500 μM p-NA.

The measured p-NA concentrations in the study samples were obtained by interpolation of their area under the curve (AUC) on the calibration curve fit. Because all the study samples reacted with identical conditions and for the same duration, a conversion of measured p-NA concentrations to activity units (amount product formed/minute reaction time/volume of plasma) was not necessary. Therefore, in this work, measured activity is expressed in micromolar amounts of product formed. This does not affect the percentage inhibition values calculated from the sample activity.

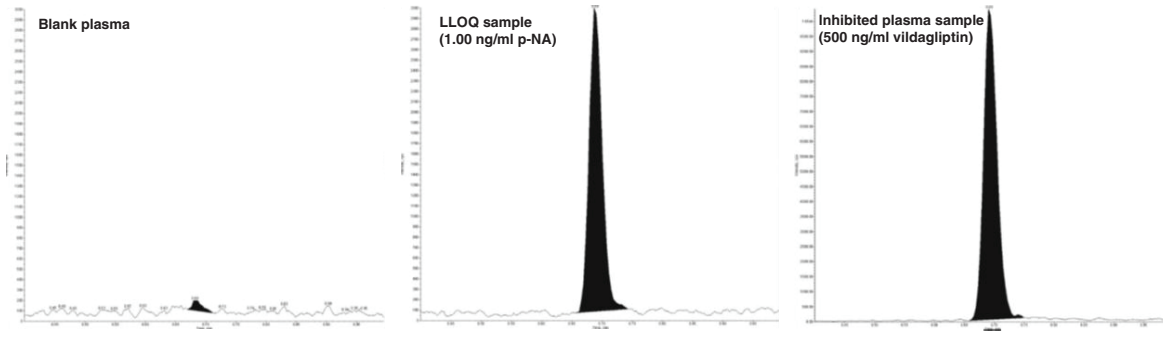

Figure 3. Chromatograms. From left to right a blank plasma sample; a lower LLOQ sample spiked at 1.00 μM para-nitroaniline (p-NA) and analyzed by adding buffer without substrate; a quality control activity QC to which a high amount of vildagliptin (500 ng/ml) was added. The p-NA measured in this sample was released by the remaining DPP-4 activity.
LLOQ: Lower limit of quantitation.

Accuracy of the method

For this method, we can define three types of accuracy.

First, there is the accuracy of the measurement of p-NA in plasma samples spiked with p-NA. This is the direct approach in which the accuracy is expressed as the bias of the measured value compared with its nominal prepared concentration. This is the approach commonly applied in regulatory guidances for validation of chromatographic methods [5,6].

Second, there is the accuracy of the amount of p-NA released by DPP-4 in a specific sample under the chosen reaction conditions. Because the activity of DPP-4 in a sample is not a value that can be set, the amount of p-NA released cannot be predicted. Therefore, instead of using a nominal concentration to compare the outcome of a measurement, a value must be established for a specific sample by averaging the results of repeated measurements of the sample. This established average value is considered to be the nominal value in later experiments. There is no specific regulatory guidance for determining and using such a QC value, although the 2018 US FDA guidelines [5] suggest this approach.

Finally, accuracy can be expressed as the correlation between the measured inhibition and the *in vivo* situation. The various commercially available substrates to monitor the progression of this reaction using colorimetric methods were designed so that a specific chromophore is released or altered by DPP-4. It is not known to what extent these interactions are comparable to those of DPP-4 substrates *in vivo*. It is known, however, that the different synthetic substrates have different affinities for DPP-4 and, furthermore, that the inhibitory effect of vildagliptin on DPP-4 differs for the different synthetic substrates; it is reasonable to assume that this is also the case for endogenous substrates. Therefore, it is crucial to evaluate the accuracy of a method in this manner to prevent the measured inhibition from misrepresenting the effect of the drug. For this type of accuracy, there are no guidelines describing acceptance criteria or experiments to be executed.

Such a misrepresentation is illustrated by the following: initially we developed a method with good performance but in which vildagliptin concentrations did not result in significant inhibition as was expected in the clinical trial samples. In this initial method, we tried to mimic physiological conditions where possible (37°C, pH, ion strength etc.), which resulted in a high reaction rate. To have sufficient substrate available to keep the reaction linear long enough for a robust measurement, the substrate concentration had to be 25,000 μM. With this method, at 1000 ng/ml vildagliptin, which is far greater than the concentrations expected in the clinical trial samples, the inhibition is only 25%, as shown in Figure 4. With such a method, only a few trial samples would show significant inhibition. Although it would still be possible to validate the method, the clinical sample results would not show a significant pharmacodynamic response to the dosed drug, which would undermine the value of the assessment of bioequivalence between the dosage forms.

Because vildagliptin is a competitive inhibitor, it competes with the substrate for interaction with DPP-4 and a higher concentration of substrate displaces its inhibitory effect to higher vildagliptin concentrations. To counter the issue, we decided to set the reaction conditions in a way that vildagliptin concentrations in which the inhibitory effect is observed correspond to those expected from the dosing in the trial [7,8]. In this concentration range,

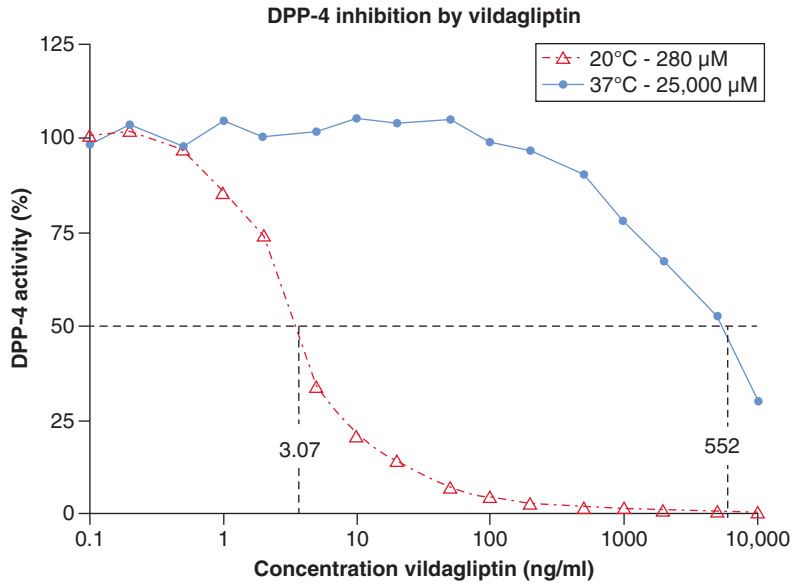

Figure 4. Effect of vildagliptin concentration on measured activity for two sets of reaction conditions. At physiological conditions, a high substrate is required, and inhibition starts at higher vildagliptin concentration, the IC_{50} is estimated at 552 ng/ml. For the lower reaction temperature and substrate concentration, the inhibition occurs in the clinically relevant concentration range and an IC_{50} of 3.07 ng/ml.

vildagliptin therapy has been demonstrated to improve glycemic control in patients [9,10]. As is shown in Figure 4, the incidence of inhibition of DPP-4 by vildagliptin is observed between 1.00 and approximately 200 ng/ml. Lower values do not show significant inhibition and higher values give close to complete inhibition. This was achieved by lowering the temperature of the reaction from 37 to 20°C which reduced the reaction rate and allowed the reaction to be linear for 15 min, sufficient for a robust measurement, while using a significantly lower concentration of substrate.

Validation

International guidelines [5,6] do not offer clear guidance for the validation experiments that cover enzymatic conversion and inhibition parameters, but they can be applied to the measurements of p-NA in plasma. Therefore, this 'fit for purpose' validation comprises two parts, the first part for which the guidelines could be applied and a second part that consists of experiments and criteria appropriate to document the characteristics and performance of the method in its entirety based on scientific rationales.

The first part of the validation follows the guidelines as deemed relevant for the method context of use. For example, whole blood stability for p-NA was not tested as p-NA is not present in blood. Plasma stability for p-NA was demonstrated to allow for the storage of spiked calibration and QC samples. Relevant experiments were performed as per the Bioanalytical Method Validation guidances [5].

For the second part, the additional experiments are listed next. The method was evaluated for both the measured DPP-4 activity and for the expected relative inhibition range calculated from the measured pre- and post-dose study sample activity values.

The experiments and their results are shown in Table 1.

Linearity of the reaction

The linearity of the reaction conditions was determined during the method development as described earlier and repeated in the validation with multiple sources of plasma from healthy volunteers. Acceptability of the results was evaluated by visual evaluation of the results (Figure 2), and no formal acceptance criteria were set.

Effect of matrix on the reaction

The DPP-4 activity varies between different plasma lots, and it is not possible to compare the measured result to a nominal value. An experiment was performed to assess the effect of matrix on several aspects of the reaction.

Table 2. Established activity and inhibition values and their variability.

	Low activity		Medium activity		High activity
	Concentration (μM)	Inhibition (%)	Concentration (μM)	Inhibition (%)	Concentration (μM)
Average	3.14	97.3	39.0	67.1	120
Total CV (%)	16.4	0.4	16.8	5.5	21.2

CV: Coefficient of variation.

Figure 2 shows that all different matrix lots are capable of reaching the plateau value at which the substrate has been depleted because after 180 min of reaction time all matrices have a highly similar response with a CV of 1.76%. Depending on the individual DPP-4 activity, the lots reach the plateau at different times. For all lots, the reaction is linear for at least up to 15 min, at which point the differences in DPP-4 activity result in a CV of 19%. The experiment includes the results from a lipemic plasma lot (triglyceride level >400 mg/dl) and a hemolytic lot (2% v/v of lysed whole blood added). Absolute activities found in clinical predose study samples were in line with the blank plasma evaluated during method validation.

Establishing activity, inhibition & acceptance criteria

Three activity samples were prepared from the same plasma lot, two were inhibited with 5.00 ng/ml and 500 ng/ml of vildagliptin, respectively, and one was used without inhibition. These samples represent low, medium and high activity, respectively. The absolute activity of DPP-4 in these samples was established by analyzing aliquots in five runs in sixfold. The results of these measurements are shown in Table 2. The average results from these determinations were used as the established activity for each sample. From the activity measurements in the inhibited and the uninhibited samples, the established inhibition values for the two inhibited samples were calculated.

The inhibition was calculated using the following formula:

$$I = 100\% \times \frac{A0 - At}{A0}$$

where $A0$ is the absolute activity (in micromolars) of the neat plasma and At is the activity in the corresponding inhibited plasma. At higher inhibition, the experimental variability on the measured residual activity (At) does not significantly impact the inhibited activity ($A0 - At$) because it is a much larger value. This results in low %CV values at high inhibition. For example, 30% of 3.14 μM is 0.942 μM; if we compare this to the inhibited amount of activity (120 μM–3.14 μM = 117 μM), this constitutes only 0.56%. For samples with lower inhibition values, the difference between At and $A0 - At$ is much smaller, and the variability in the measured residual activity has more impact on the variability observed in the inhibition (I) results. The experimental variability in ($A0$) is mostly canceled out by this calculation.

For activity measurements, this observation allows us to accept a higher variability than what is commonly accepted in validations of chromatographic biomarker methods because it does not propagate to the variability in the calculated inhibition values. As can be seen in Figure 5, the majority of samples in the curve have a relatively high inhibition, except the predose samples. On the basis of the results of the experiment, the acceptance criterion for variability in absolute activity measurements of 30% CV is deemed appropriate.

Accuracy & precision

In an accuracy and precision experiment, three runs were performed in which samples were measured in sixfold. The bias of each individual measurement from the established nominal value was calculated, as well as the variability in the resulting inhibition values. Acceptance criteria of 30% were used as mentioned earlier.

Stability of activity & inhibition

The three plasma activity QC samples were analyzed after storage at -20 and -70°C, after overnight storage on the benchtop and after five freeze–thaw cycles. The measured values were compared with the established activity value to assess the stability of DPP-4. The percentage inhibition was also used to assess the impact on the interaction between DPP-4 and vildagliptin.

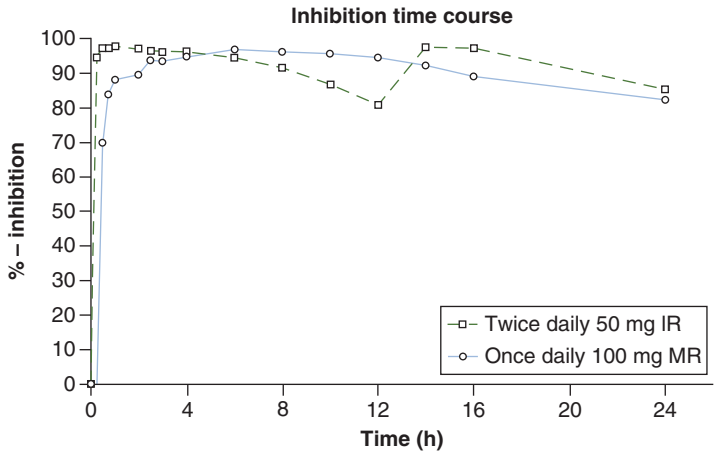

Figure 5. Inhibition profiles of two subjects. One subject was dosed twice daily and one was dosed once with the new modified release.

Bioequivalence trial results

To illustrate the results of the analysis of the bioequivalence trial samples, Figure 5 shows the percentage inhibition of two representative subjects, one dosed orally twice daily with 50 mg of vildagliptin immediate-release formulation and the other with once-daily 100-mg vildagliptin as a modified-release formulation. The inhibition profiles show minor differences, but AUC values from 0 to 24 h are not statistically different; there is only 2.5% difference for these subjects. This is in line with conclusions after analysis of the complete dataset with all trial subjects: the inhibition for the new dosage form is equivalent to the original form. The evaluation of the complete dataset was published with the other results of the trial [11].

During the analysis of the bioanalytical samples from the clinical trial, several QC samples showed absolute activity values that were out of specification (i.e., having a bias from the established value). However, the corresponding inhibition values calculated for these QCs by dividing their measured activity by the activity measured in the matched predose samples were all within acceptance criteria. Because the primary endpoint of the trial was inhibition rather than absolute enzyme activity, we suggest that for similar assays no criteria for QCs be set for absolute activity but only for inhibition.

Conclusion

An LC-MS/MS-based method for the determination of the activity and inhibition of DPP-4 was developed. The characteristics and performance of the method met predefined acceptance criteria, and the method was validated and its performance deemed fit for the purpose of assessing bioequivalence. In total, 2158 samples were measured in the context of the trial, and their results showed bioequivalence of the two formulations.

Recommendations

To compensate for the lack of a direct method to determine the accuracy of inhibition measurements, method development scientists should evaluate whether the reported inhibition for a given concentration of inhibitor correlates with the normal range of plasma concentrations of dosed subjects, or, in the case of a drug with documented pharmacokinetic properties, preferably with the occurrence of the therapeutic effect of the drug.

Variability in the measurement of the absolute enzyme activity in inhibited samples measured as amount of product formed does not significantly transfer to the error in the determined percent inhibition. For trials in which inhibition is of primary interest, we conclude that criteria should only be set for the inhibition of QCs and not for the absolute activity values required for its calculation.

Future perspective

As an analytical platform, liquid chromatography and tandem mass spectrometry (LC-MS/MS) has several advantages over colorimetry, which still is a common detection method for activity measurements. These include a higher sensitivity, a larger linear range, less interference from matrix components, the use of internal standards and a more specific mode of detection. A large advantage of colorimetric methods is that they are significantly less expensive, although LC-MS/MS is quickly becoming a more common analytical platform. Considering the ever-increasing regulatory scrutiny on bioanalysis and the constant drive of the pharmaceutical industry to deliver higher quality,

the authors speculate that in coming years, there might well be an increase in the number of activity and inhibition assays performed with LC-MS/MS.

Summary points

- An analytical method based on liquid chromatography and tandem mass spectrometry (LC-MS/MS) enzyme conversion product detection was developed, validated and applied to the measurement of the inhibition of DPP-4 in human plasma samples from a bioequivalence trial.
- The activity of DPP-4 is determined by measuring the concentration of p-NA, which is formed when DPP-4 interacts with its substrate, GP-p-NA.
- The inhibition is calculated by comparing the residual absolute enzyme activity following a dose of DPP-4 inhibitor to the predose activity in the same subject.
- The fit for purpose validation encompasses many experiments based on international guidelines but also includes experiments to extend the validated state to the reaction between DPP-4 and its substrate GP-p-NA based on scientific judgment.
- The analysis of human trial samples indicated that two dosage forms of DPP-4 inhibitor (vildagliptin) are bioequivalent.

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Financial & competing interests disclosure

The authors are employees of either ICON plc or Novartis. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

Papers of special note have been highlighted as: • of interest

1. Ahrén B. DPP-4 inhibition and the path to clinical proof. *Front. Endocrinol. (Lausanne).* 10(JUN), 1–18 (2019).
2. Holst JJ, Deacon CF. Inhibition of the activity of dipeptidyl-peptidase IV as a treatment for type 2 diabetes. *Diabetes* 47(11), 1663–1670 (1998).
3. Herman GA, Bergman A, Stevens C *et al.* Effect of single oral doses of sitagliptin, a dipeptidyl peptidase-4 inhibitor, on incretin and plasma glucose levels after an oral glucose tolerance test in patients with type 2 diabetes. *J. Clin. Endocrinol. Metab.* 91(11), 4612–4619 (2006).
4. Ma H, Qian XK, Zhang J *et al.* Accurate and sensitive detection of dipeptidyl peptidase-IV activity by liquid chromatography with fluorescence detection. *Anal. Methods* 12(6), 848–854 (2020).
   - **Introduces the use of chromatographic separation to the detection of DPP-4 activity.**
5. Center for Drug Evaluation and Research, US FDA. Bioanalytical Method Validation, Guidance for Industry (2018). https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf
6. EMA, Committee for Medicinal Products for Human Use. Guideline on bioanalytical method validation (2011). https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf
7. Hu P, Yin Q, Deckert F *et al.* Pharmacokinetics and pharmacodynamics of vildagliptin in healthy Chinese volunteers. *J. Clin. Pharmacol.* 49(1), 39–49 (2009).
   - **Includes a good comparison of the pharmacokinetics of vildagliptin and its effect on the activity of DPP-4.**
8. He Y-L. Clinical pharmacokinetics and pharmacodynamics of vildagliptin. *Clin. Pharmacokinet.* 51(3), 147–162 (2012).
9. Panina G. The DPP-4 inhibitor vildagliptin: robust glycaemic control in type 2 diabetes and beyond. *Diabetes. Obes. Metab.* 9(Suppl. 1), 32–9 (2007).

10. Kikuchi M, Abe N, Kato M *et al*. Vildagliptin dose-dependently improves glycemic control in Japanese patients with type 2 diabetes mellitus. *Diabetes Res. Clin. Pract.* 83(2), 233–240 (2009).

11. Sangana R, Mittal H, Barsainya S *et al*. Therapeutic equivalence of vildagliptin 100 mg once daily modified release to 50 mg twice daily immediate release formulation: an open-label, randomized, two-period, single- and multiple-dose, 6-day crossover study.. *Diabetes Metab. Syndr. Clin. Res. Rev.* (2022) (In Press).

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Commentary

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Bioanalysis

Large molecule bioanalysis by LC–MS: beyond simply quantifying

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> "Before developing a method, it should be clear what the exact question that needs to be answered and what kind of concentration result offers most value in a particular research or health care context."

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During the last decade, the quantitative bioanalysis of biopharmaceuticals and large molecule biomarkers by liquid chromatography–mass spectrometry (LC–MS) has been an important research topic in our group. Since we, and many others, started asking ourselves whether LC–MS could make a difference for targeted protein quantification [1], the world has seen important developments in the technology. LC–MS can now definitely be regarded as an established platform for protein bioanalysis, next to the traditional ligand-binding assay (LBA), and it is routinely used to support academic research, industrial drug development and hospital care. In the early years, most of the research efforts were focused on the technological aspects of protein quantification, to develop approaches capable of equaling or exceeding the performance of LBAs in terms of sensitivity, selectivity, precision and accuracy. The typical workflow was, and often still is, a combination of extracting the protein of interest from the sample, enzymatically digesting it to a series of peptides and quantifying one or more of these, the so called signature peptides, by LC–MS along with a suitable protein based or peptide based internal standard for response normalization [2]. With limited detection sensitivity being the main technical drawback of LC–MS compared to LBA, an important research area was the development of optimal sample preparation methods to decrease sample complexity and thereby improve detectability [3]. In our group, we concentrated on antibody free protein extraction approaches [4–7], to avoid the potential limitations in availability and consistency of critical immunological reagents and to make full use of LC–MS as an orthogonal method to LBA, although for ultimate selectivity and sensitivity analyte specific enrichment, using anti-idiotypic antibodies for example, often remains the preferred option [8,9]. By properly combining different analytical tools, LC–MS based protein quantification in complex biological samples can now be routinely and reliably achieved down to the pg/ml level and has, thus, become a viable alternative to analysis by LBA.

With the advancement of the technology, it also became evident that LC–MS quantification is less straightforward for a protein than for a small molecule. Proteins occur *in vivo* in their free form and/or are bound to one or more endogenous binding partners, and it depends on the principle of the analytical method whether a total, free, or bound concentration is obtained, or indeed something in between [10,11]. In addition, it is an oversimplification to consider the protein analyte as a homogeneous and inert molecule with a single, well defined concentration. On the contrary, proteins often exist as sets of isoforms with different chemical and 3D structures, each of which may or may not undergo structural changes during their residence in the body. Since both LC–MS and LBA are directed to small and sometimes different parts of these multiple protein molecules, it is not uncommon that different analytical approaches yield different concentration results for the same sample [12]. Therefore, our research increasingly focuses on the occurrence of protein heterogeneity *in vivo* and the required analytical tools, from

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sample preparation to separation and detection, to study its quantitative significance. This commentary discusses these aspects in some more detail.

Protein heterogeneity & biotransformation

Proteins that are derived from a single DNA sequence constitute families of molecules due to post-translational modifications, proteolytic processing or degradation and chemical modifications (e.g., deamidations). In addition, heterogeneity may be due to the design of the therapeutic protein, for example, because of different drug-to-antibody ratios (DARs) for antibody–drug conjugates (ADCs). As outlined above, commonly used bioanalytical assays based on LBAs or LC–MS target only a small part of the protein and thus generate a single concentration value for a given protein. This result must, however, be viewed in the context of what the assay actually measures (e.g., a given epitope or signature peptide), and it should be realized that it captures only a certain part of the protein family.

Although protein heterogeneity has been extensively studied as part of the quality control of recombinant, therapeutic proteins, much less is known about the fate of these proteins once administered to patients, which is often referred to as *in vivo* biotransformation. While it is unlikely that toxic metabolites are formed from proteins, in contrast to some small molecule drugs, *in vivo* biotransformation may affect their pharmacokinetics and pharmacodynamics or activity. LBAs and LC–MS assays can be affected by such transformations. The assay response may either be lost due to a given biotransformation or it may not be affected at all. It is thus important to know which part of a protein is targeted by a bioanalytical assay and to design the assay in such a way that relevant information is obtained. When studying the *in vivo* biotransformation of the biopharmaceutical drug trastuzumab in patients, we observed that an LC–MS assay with two different signature peptides (one stable peptide and one with a deamidation site), a receptor binding assay and an immunoassay using anti-idiotypic antibodies gave vastly different results, depending on the deamidation of an asparagine residue in the target binding region of the drug [13]. In a preclinical study with recombinant somatropin being dosed to rats, an LC–MS method with three different signature peptides showed a good correlation between the concentrations of two of these in all samples, but a significantly higher concentration for the third peptide, which suggests that the part of the protein containing this peptide is less susceptible to enzymatic and chemical modifications *in vivo* than the parts comprising the other two peptides [14].

With the appearance of novel constructs for therapeutic proteins, such as ADCs, bispecific antibodies and pegylated proteins, it is more than ever necessary to use properly designed assays that capture the novel features of these drugs. LC–MS assays are likely better suited than LBAs to reach this goal in a reasonable timeframe, by selecting and quantifying signature peptides or other chemical entities that represent the feature in question.

Sample preparation

Protein extraction is an important step in the bioanalysis of therapeutic proteins. When these are present at relatively high concentrations (high ng/ml levels or above), isolation and subsequent digestion of the entire protein fraction from, for example, plasma or serum often provide sufficient selectivity and sensitivity. In such a case, all different isoforms of the protein of interest will be extracted from the sample and can be further quantified. For studying the heterogeneity of biopharmaceuticals or biomarkers at lower concentrations, some form of selective extraction is needed to remove interferences originating from the enormous excess of endogenous proteins. However, the use of commercially available or tailor-made immunoaffinity reagents against one form of a protein cannot capture all its isoforms. For example, the *in vivo* deamidation of trastuzumab and pertuzumab was found to cause a complete loss of recognition by both their target receptor and an anti-idiotypic antibody [15]. Similarly, an antibody directed to the major circulating form of the biomarker human growth hormone showed less affinity toward three other less abundant isoforms, resulting in up to threefold lower extraction recoveries [data not published]. This shows that a single affinity reagent may not be sufficient for capturing the multiple forms of proteins *in vivo.*

The advent of non-antibody based binders opens new possibilities in this respect [16]. Most of them, such as Affimers, are selected with a display technology (e.g., phage display) against the target protein, so they only respond to the protein that is used in the screen. This means, for example, that if a given target protein used for screening does not contain the recognition element that is needed for following an *in vivo* biotransformation, it will be impossible to find an appropriate binder for the biotransformation product. An Affimer reagent that was selected because of its good binding properties toward trastuzumab showed a significant decrease in extraction recovery when trastuzumab was incubated at pH 7.4 and 37°C for 3 weeks to mimic *in vivo* conditions, while a similar

treatment of pertuzumab did not lead to reduced extraction efficiency by its own optimal Affimer [17]. This could be due to a much-reduced recognition by the trastuzumab binder of the various biotransformation products that were formed upon incubation, while the pertuzumab binder might still be able to capture the modified forms of this biopharmaceutical if these were formed at all. It also indicates that the use of multiple binders against different epitopes might be required to obtain a more complete picture of the *in vivo* fate of different proteoforms.

Separation & detection

An additional challenge for the quantification of multiple forms of protein analytes is fact that it may be difficult, or even impossible, to find signature peptides that are unique for the different isoforms and, thus, to separately quantify them in each other's presence. It is also increasingly recognized that a signature peptide may represent as little as a few percent of the entire protein, and that the structural information about the rest of the protein molecule is therefore automatically lost. Consequently, there is a growing desire to quantify proteins in their intact form, and this asks for additional developments in technology [18]. The interaction of biopharmaceuticals with endogenous binding proteins can be studied with size-exclusion chromatography because of the usually large difference in size between the free and protein bound forms of the analyte [19]. The separation of unbound, intact proteins is most often achieved by reversed phase (RP) LC, and different hydrophobic column packings with pore sizes up to 1000 Å are available to allow selection of an optimal stationary phase for a wide range of proteins. Still, the differences between the isoforms of a particular protein in terms of size and hydrophobicity are typically small, and it is often difficult to accomplish sufficient chromatographic resolution with RP–LC.

A more efficient alternative is ion-exchange (IEX) LC, which separates intact proteins based on differences in charge, caused by, for example, the deamidation of a neutral asparagine to an acidic aspartate in the primary structure of a protein [20]. To give an example, a cation-exchange column in combination with a 70-min mobile phase pH gradient allowed the high-resolution separation of about 20 isoforms (charge variants) of trastuzumab, which were formed from the original drug after 3 weeks at physiological conditions. Mobile-phase fractions corresponding to the charge variants were collected and further characterized by tryptic digestion and LC–MS-based peptide mapping [21]. A disadvantage of this approach is the typical presence of high concentrations of non-volatile buffer salts in the mobile phase, which complicates the online coupling of IEX–LC to MS detection, and more research into the technical possibilities will be necessary to improve its use for protein quantification.

The MS detection of intact proteins also is more complicated than that of their often relatively small signature peptides. Electrospray ionization of large proteins gives rise to complex mass spectra because the ionized protein is present in several forms with varying numbers of charges across the molecule. Each of these charge states is further subdivided into multiple ions with different mass-to-charge ratios because of the natural occurrence of heavy isotope forms of some atoms. By high-resolution mass spectrometry (HRMS), based on, for example, quadrupole–time of flight instrumentation, the detection responses of each of these many ions can be recorded, and so-called extracted ion chromatograms can be constructed by combining the intensities of multiple ions. This technique is traditionally used for qualitative purposes, and its detection sensitivity is often perceived as less favorable than that of the standard triple-quadrupole mass spectrometer. However, its usefulness for intact protein quantification at trace levels in biological samples has been demonstrated, such as for the 22 kDa biopharmaceutical somatropin, which could be quantified down to 10 ng/ml in rat plasma, when combined with immunoaffinity extraction [22]. Here, it should be noted that when protein isoforms have small or no differences in mass, HRMS may not be able to distinguish the different forms, and some sort of chromatographic resolution is essential to separate the different forms prior to their detection.

Conclusion

After more than a decade of developments, protein quantification by LC–MS is transitioning into a new phase. The focus is no longer solely on the methodology, i.e., how a concentration result can be obtained in the simplest way, but increasingly also on more conceptual aspects. Before developing a method, it should be clear what the exact question that needs to be answered and what kind of concentration result offers most value in a particular research or health care context. Is total, free, or bound protein the most relevant; one, several, or the sum of all isoforms; and which part or parts of the molecule(s) are most important to target? Only when this is known can a method, be it LC–MS or LBA for intact or digested analytes, be designed in such a way that one or more relevant concentration results are unambiguously obtained. Many analytical tools are already available, but we expect that further research remains necessary to help develop this important and fascinating field.

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References

1. van de Merbel NC, Bronsema KJ, Nemansky M. Protein quantification using LC–MS: can it make a difference? *Bioanalysis* 4(17), 2113–2116 (2012).
2. Bronsema KJ, Bischoff R, van de Merbel NC. High-sensitivity LC–MS/MS quantification of peptides and proteins in complex biological samples: the impact of enzymatic digestion and internal standard selection on method performance. *Anal. Chem.* 85(20), 9528–9535 (2013).
3. Bischoff R, Bronsema KJ, van de Merbel NC. Analysis of biopharmaceutical proteins in biological matrices by LC–MS/MS – I. Sample preparation. *Trends Anal. Chem.* 48, 41–51 (2013).
4. Wilffert D, Reis CR, Hermans J *et al.* Antibody-free LC–MS/MS analysis of recombinant tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in human and mouse serum. *Anal. Chem.* 85(22), 10754–10760 (2013).
5. Bronsema KJ, Bischoff R, Bouche MP, Mortier K, van de Merbel NC. High-sensitivity quantification of a nanobody in plasma by single-cartridge multi-dimensional SPE and ultra-performance LC–MS/MS. *Bioanalysis* 7(1), 53–64 (2015).
6. Wilffert D, Bischoff R, van de Merbel NC. Antibody-free workflows for protein quantification by LC– MS. *Bioanalysis* 7(6), 763–779 (2015).
7. Wilffert D, Donzelli R, Asselman A *et al.* Quantitative antibody-free LC–MS/MS analysis of sTRAIL in sputum and saliva at the sub-ng/ml level. *J. Chromatogr. B.* 1032, 205–210 (2016).
8. Klont F, Pouwels SD, Hermans J *et al.* A fully validated liquid chromatography-mass spectrometry method for the quantification of the soluble receptor of advanced glycation end-products (sRAGE) in serum using immunopurification in a 96-well plate format. *Talanta* 182, 414–421 (2018).
9. Klont F, Pouwels SD, Bults P *et al.* Quantification of surfactant protein D (SPD) in human serum by liquid chromatography-mass spectrometry (LC–MS). *Talanta* 202, 507–513 (2019).
10. Bronsema KJ, Bischoff R, Pijnappel WWMP, van der Ploeg AT, van de Merbel NC. Absolute quantification of the total and antidrug antibody-bound concentrations of recombinant human α-glucosidase in human plasma using protein G extraction and LC–MS/MS. *Anal. Chem.* 87(8), 4394–4401 (2015).
11. van de Merbel NC. Protein quantification by LC–MS: a decade of progress through the pages of Bioanalysis. *Bioanalysis* 11(7), 629–644 (2019).
12. Bults P, van de Merbel NC, Bischoff R. Quantification of biopharmaceuticals and biomarkers in complex biological matrices: a comparison of liquid chromatography coupled to tandem mass spectrometry and ligand binding assays. *Expert Rev. Proteomics* 12, 355–374 (2015).
13. Bults P, Bischoff R, Bakker H, Gietema JA, van de Merbel NC. LC–MS/MS based monitoring of *in vivo* protein biotransformation: quantitative determination of trastuzumab and its deamidation products in human plasma. *Anal. Chem.* 88(2), 1871–1877 (2016).
14. Bults P, Meints M, Sonesson A, Knutsson M, Bischoff R, van de Merbel NC. Improving selectivity and sensitivity of protein quantitation by LC-HR-MS/MS: determination of somatropin in rat plasma. *Bioanalysis* 10(13), 1009–1021 (2018).
15. Bults P, van der Voort A, Meijer C, Sonke GS, Bischoff R, van de Merbel NC. Analytical and pharmacological consequences of the *in vivo* deamidation of trastuzumab and pertuzumab. *Anal. Bioanal. Chem.* 414, 1513–1524 (2022).
16. Olaleye O, Govorukhina N, van de Merbel NC, Bischoff R. Non-antibody-based binders for the enrichment of proteins for analysis by mass spectrometry. *Biomolecules* 11(12), 1791 (2021).
17. Olaleye O, Spanov B, Ford R, Govorukhina N, van de Merbel NC, Bischoff R. Enrichment and liquid chromatography-mass spectrometry analysis of trastuzumab and pertuzumab using Affimer® reagents. *Anal. Chem.* 93(40), 13597–13605 (2021).
18. Bults P, Spanov B, Olaleye O, van de Merbel NC, Bischoff R. Intact protein bioanalysis by liquid chromatography – high-resolution mass spectrometry. *J. Chromatogr. B.* 1110–1111, 155–167 (2019).
19. Spanov B, Aboagye V, Olaleye O, Govorukhina N, van de Merbel NC, Bischoff R. Effect of trastuzumab-HER2 complex formation on stress-induced modifications in the CDRs of trastuzumab. *Front. Chem.* 9, 794247 (2022).
20. Spanov B, Govorukhina N, van de Merbel NC, Bischoff R. Analytical tools for the characterization of deamidation in monoclonal antibodies. *J. Chromatogr. Open* 2, 100025 (2022).
21. Spanov R, Olaleye O, Lingg N *et al.* Change of charge variant composition of trastuzumab upon stressing at physiological conditions. *J. Chromatogr. A.* 1655, 462506 (2021).
22. Bults P, Sonesson A, Knutsson M, Bischoff R, van de Merbel NC. Intact protein quantification in biological samples by liquid chromatography – high-resolution mass spectrometry: somatropin in rat plasma. *J. Chromatogr. B.* 1144, 122079 (2020).

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