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Intact protein quantification in biological samples by liquid chromatography – high-resolution mass spectrometry: somatropin in rat plasma

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ABSTRACT

The quantitative determination of intact proteins in biological samples by LC with high-resolution MS detection can be a useful alternative to ligand-binding assays or LC-MS-based quantification of a surrogate peptide after protein digestion. The 22-kDa biopharmaceutical protein somatropin (recombinant human growth hormone) was quantified down to 10 ng/mL (0.45 nM) in 75 μL of rat plasma by the combination of an immunocapture step using an anti-somatropin antibody and LC-MS on a quadrupole-time of flight instrument. Accuracy and precision of the method as well as its selectivity and sensitivity did not depend on the width of the mass extraction window nor on whether only one or a summation of multiple charge states of the protein analyte were used as the detection response. Quantification based on deconvoluted mass spectra showed equally acceptable method performance but with a less favorable lower limit of quantification of 30 ng/mL. Concentrations in plasma after dosing of somatropin to rats correlated well for the deconvolution approach and the quantification based on the summation of the response of the four most intense charge states (14^+ to 17^+) of somatropin.

1. Introduction

Over the past decade, there have been many developments in the field of targeted protein quantification by liquid-chromatography-mass spectrometry (LC-MS) and this technique has become a viable alternative to the traditional ligand-binding assays (LBAs). Analytical advantages of LC-MS for protein quantification include superior accuracy and precision, a wider linear dynamic range, the potential to measure multiple analytes simultaneously and no, or at least a lesser, dependence on critical immunochemical reagents that may be difficult to obtain and/or vary in quality. The major drawback of LC-MS compared to LBA is its limited detection sensitivity. Consequently, complicated workflows may be needed to allow protein quantification at trace levels in complex matrices. In particular, an enzymatic digestion step to convert a protein into a series of peptides is part of most LC-MS methods, to reduce the macromolecular analyte to a size that can readily and sensitively be quantified by MS/MS detection on a triple-quadrupole mass spectrometer [1–6].

Although this quantification approach via a so-called signature or surrogate peptide is being applied with much success in the medical and

pharmaceutical sciences, it is increasingly recognized that it also has some disadvantages. The selected signature peptide may represent as little as a few percent of the original protein analyte and since the rest of the molecule is disregarded, important information about the *in vivo* fate of a dosed or endogenously occurring protein may be lost. This can be addressed to some extent by including multiple peptides from relevant parts of the protein in an LC-MS assay [7], but quantification of the intact protein would be the most direct and most comprehensive approach.

In recent years, the proof of principle of intact protein quantification by LC-MS has been demonstrated, typically by using high-resolution mass spectrometry (HRMS) on an Orbitrap or quadrupole-time of flight (QTOF) system. Initially, the approach was restricted to relatively small (< 15 kDa) proteins [8], but more recently molecules as large as protein domains [9] and even intact monoclonal antibodies [10,11] were successfully quantified in biological samples at the low-μg/mL to high-ng/mL level. Intact protein quantification requires a quite different analytical approach than analysis after digestion [12]. Next to the need for specific LC stationary phases to separate the intact protein species, selective extraction from the biological matrix by means of

immunocapture techniques is indispensable to avoid interference from endogenous matrix proteins. In addition, data handling is more complicated. Electrospray ionization (ESI) leads to complex mass spectra because the ionized protein analyte occurs in multiple charge states (the so-called charge-state envelope). The number of different charge states depends on the size and shape of the protein as well as on the ionization conditions; for large proteins such as antibodies, it can be as high as 30 [10,11]. Each of the charge states is further subdivided into several ions with different mass-to-charge (m/z) ratios because of the occurrence of different numbers of heavy isotopes, notably ^{13}C-atoms, in the protein structure, and these isotopologue ions may or may not be mass spectrometrically resolved. All this results in a distribution of the MS-signal over a large number of analyte ions with different m/z values, which leads to a limited detection response per ion. Typically, so-called extracted ion chromatograms (EIC's) are created by recording the responses of one or more ions and detection sensitivity can be enhanced by summing up the intensities of multiple ions. Alternatively, dedicated software can be applied to deconvolute the entire mass spectrum into a much-simplified neutral spectrum, which can be used as the basis for quantification. Since all approaches have their theoretical pros and cons, careful optimization is required to arrive at a method that shows acceptable sensitivity, selectivity, precision and accuracy [13].

To support preclinical research with the 22-kDa recombinant protein somatropin in rats, we previously developed and validated an LC-HRMS method which included a digestion step and quantification of three peptides from different parts of the molecule [14]. In the present report, we describe a complementary method which enables quantification of the intact protein in rat plasma down to 10 ng/mL. The importance of an immunocapture step and the data handling approach (selection of charge states and deconvolution) is described, and a comparison of the pharmacokinetic results for intact somatropin obtained after quantitation based on extracted ion chromatograms and obtained after deconvolution is provided.

2. Experimental

2.1. Chemicals and materials

Somatropin (recombinant human growth hormone, rhGH; UniProtKB ID 'P01241'; Phe27- Phe217), supplied as a lyophilized sterile powder, was obtained from Ferring (Copenhagen, Denmark), its amino acid sequence is given in Figure S-1 (supplementary information). The internal standard, N-terminal His-tag labelled rhGH (Cat. No. ABIN1719793; six histidine moieties) was purchased from Antibodies-online (Aachen, Germany). The capture antibody, mouse anti-human growth hormone monoclonal antibody (Cat. No. ab9821), was obtained from Abcam (Cambridge, United Kingdom). A protein biotin labelling kit (Cat. No. 11418165001; Roche) was purchased from Sigma Aldrich (St. Louis, MO, USA). Pierce™ streptavidin-coated magnetic beads (Cat No. 88817) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Rat growth hormone binding protein (Cat. No. CYT-933) was purchased from ProSpec Protein Specialists (Ness-Ziona, Israel) and catalase (2000–5000 units/mg, Cat. No. C9322) from Sigma Aldrich. Acetonitrile, formic acid, ammonia (25%) and hydrochloric acid (37%) were obtained from Merck (Darmstadt, Germany) and Tween-20, citric acid, sodium chloride, hydrogen peroxide (30%) and Tris from Sigma Aldrich. HPLC grade water was prepared using a water purification system from Merck-Millipore. Rat EDTA plasma (Sprague Dawley, hereafter referred to as (blank) rat plasma) was obtained from Seralabs (Haywards Heath, UK). ESI positive calibration solution for the Q-TOF system (Cat No. 4463272) was obtained from Sciex (Toronto, Canada). Oxidized somatropin was prepared in house by incubating a mixture of 475 μL 1.00-μg/mL somatropin solution in water and 25 μL 5% hydrogen peroxide at 20 °C for up to 165 min. Excess hydrogen peroxide was subsequently removed from the sample by adding 25 μL catalase solution (the equivalent of 500 units) and further incubating for 5 min

at 20 °C.

2.2. Preparation of calibration and 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 (VWR International, Amsterdam, The Netherlands) and stored at −80 °C. Two plasma stocks were prepared by diluting the stock solution to 10,000 ng/mL with blank rat plasma. One plasma stock was used to prepare calibration samples in rat plasma at 10.0, 20.0, 50.0, 100, 200, 500, 800 and 1000 ng/mL. Similarly, quality control (QC) samples were prepared in rat plasma from the other stock at 30.0, 100 and 500 ng/mL. All samples were stored in Eppendorf Protein Lo-bind tubes at −80 °C.

2.3. Pharmacokinetic study

After obtaining ethical approval, Sprague Dawley rats were dosed with a single subcutaneous bolus injection of 2 mg/kg somatropin (Zomacton®). Blood samples were collected in K$_3$-EDTA tubes before and 0.5, 1, 2, 4, 8 and 24 h after dosing. Plasma was prepared immediately after blood collection and transferred to −80 °C until analysis.

2.4. Sample pretreatment

The capture antibody was biotinylated according to the instructions provided with the protein biotin labelling kit. Briefly, 300 μL anti-human rhGH antibody (1.00 mg/mL in PBS with 0.1% sodium azide, pH 7.4) was incubated at room temperature and protected from light, at 250 rpm for two hours with 20.0 μL freshly prepared Biotin 7-NHS labelling solution (0.710 mg/mL in DMSO). After incubation, the mixture was purified by running it over a Sephadex G-25 gel filtration column. The concentration of the biotinylated antibody was calculated by measuring the optical density (280 nm) of the purified solution against a corresponding blank solution and found to be 181 μg/mL. The biotinylated antibody was stored in 100 μL aliquots in Protein Lo-bind tubes at −80 °C.

Sample analysis was performed as follows. Aliquots of 75 μL of rat plasma were pipetted into the 500-μL wells of an Eppendorf Protein Lo-bind 96-well plate (VWR International) and 50 μL of a freshly diluted capture antibody solution, at 10.0 μg/mL in water, was added. Next, 200 μL of immunocapture buffer (75 mM NaCl and 8.4 mM aqueous Tris buffer at pH 7.2), containing 100 ng/mL of internal standard (His-tag labelled rhGH) was added to each of the samples. The samples were incubated at 45 °C and 900 rpm for 60 min using an Eppendorf (Hamburg, Germany) Thermomixer® comfort, to allow binding of somatropin and internal standard to the biotinylated capture antibody. Simultaneously, 15-μL aliquots of the streptavidin-coated magnetic bead solution were washed twice with 200 μL 0.2% Tween-20 in immunocapture buffer, in a separate Protein Lo-bind 96-well plate. The magnetic beads were isolated by letting the plate stand for 8 min on a 96-well magnet plate from Alpaqua Magnum FLX (Beverly, MA, USA) and removing the wash solution. Next, the samples were transferred to the plate containing the washed magnetic beads and subsequently incubated for 90 min at 45 °C and 900 rpm, using a Thermomixer®, to allow capturing of the antibody-somatropin complex by the magnetic beads. The beads were washed twice with 300 μL immunocapture buffer and once with 300 μL water. Somatropin and internal standard were eluted by mixing the beads for 10 min with 60 μL elution buffer (0.1 M citric acid in water:acetonitrile (90:10, v/v)) at 45 °C and 900 rpm, using a Thermomixer®, and subsequent capture of the beads for 5 min (total elution time: 15 min). The eluates were transferred into 300-μL glass vials and placed in an autosampler or refrigerator at 10 °C

until analysis.

2.5. Chromatography

Processed samples were analyzed using a NexeraX2 (Shimadzu, Tokyo, Japan) LC system. Chromatographic separation was performed at 80 °C on a 2.1 × 100 mm (particle size 1.7 μm, pore size 300 Å) ACQUITY UPLC Protein BEH C4 column (Waters, Milford, MA, USA, Cat No. 186004496). Mobile phase A consisted of 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Gradient elution was performed at 0.6 mL/min using the following profile: 0.0–10.0 min: 10–45% B; 10.0–10.5 min: 45–85% B; 10.5–12.5 min: 85% B; 12.5–12.6 min: 85–10% B; 12.6–15.0 min: 10% B. The injection volume was 40 μL. The mobile phase was diverted to waste between 0 and 6.5 min and between 11.5 and 15.0 min, after injection, using a VICI (Houston, TX, USA) switching valve, placed between the column and the mass spectrometer.

2.6. Mass spectrometry

Detection took place on a Sciex TripleTOF 6600 Q-TOF mass spectrometer equipped with a Dual Spray source, which was operated in positive-ion TOF MS mode at a resolution of approximately 40,000 at *m/z* 829. Analyst TF software 1.7.1 in combination with MultiQuant™ 2.1.2 including Research Features Software for charge state deconvolution, PeakView™ 3.0.2 and BioPharmaView™ 3.0 (Sciex) were used for data acquisition and processing. For the MS-system the following optimum settings were used: ionspray voltage at 5500 V, source temperature at 600 °C, declustering potential at 30 V, curtain, nebulizer and drying gas at 35 psi, 50 psi and 50 psi, respectively. All mass spectra were recorded over the range of *m/z* 550 to *m/z* 2100. The system was calibrated by application of the ESI positive calibration solution through the calibrant delivery system unit prior to every batch and during analysis after every 6 injections. Extracted ion chromatograms were created by the extraction and subsequent summation of the 14⁺, 15⁺, 16⁺ and 17⁺ charge states of somatropin, each with a mass extraction window (MEW) of ± 0.5 Da around the target mass, details of which are presented in Table 1. Deconvolution was performed using the integrated, maximum entropy algorithm within MultiQuant™. The deconvolution parameters used are shown in Supplementary Figure S-2. The resulting main peak in the reconstructed average mass spectrum was subsequently integrated and the ratio of its area over that of the similarly deconvoluted internal standard was used for further quantification.

3. Results and discussion

3.1. Immunocapture performance

For the selective LC-MS-based quantification of trace levels of intact proteins in complex biological matrices such as plasma and serum, it is important that samples are sufficiently cleaned up, to avoid

interference of endogenous matrix proteins, many of which are present at much higher concentrations than the analytes. Conventional techniques such as solid-phase extraction (SPE), based on reversed-phase or ion-exchange principles, typically do not provide enough discrimination between endogenous proteins and the analyte for detection at the ng/mL level in plasma, which was required for supporting the preclinical study with somatropin in rats. Therefore, a more selective immunocapture (IC) step was optimized using a commercially available anti-somatropin monoclonal antibody. Because of its practical ease of use and high sample throughput, a magnetic-bead-based approach in a 96-well format was selected. The capture antibody was biotinylated and after incubation with sample, the resulting complex was captured by streptavidin-coated beads, which results in an IC complex that is very strongly bound to the beads, because of the high affinity of the biotin-streptavidin interaction [5]. The extraction was optimized with regard to amount of antibody and magnetic beads, duration and temperature of the capture and elution steps, and the composition of the wash and elution solvents (see Table S-1 in the supplementary materials for details).

Although various other IC approaches exist [15,16], in our hands pre-incubation of 75 μL of rat plasma with 50 μL of a capture antibody solution (10 μg/mL) and an incubation step, followed by subsequent addition of 15 μL of magnetic beads solution and a second incubation step, resulted in high and reproducible extraction recoveries (> 80%) with good linearity. This indicates that the amounts of IC materials used had adequate binding capacity. Endogenous plasma constituents, which could possibly interfere at the retention time of somatropin, were sufficiently removed, as judged from the chromatograms obtained for blank rat plasma (Fig. 1). Of note is the need to include Tween-20 during part of the sample preparation to counter the negative effects of non-specific binding of somatropin to the sample preparation materials. To avoid interference of Tween-20 in the subsequent LC-MS assay, a final wash step with pure water to eliminate Tween-20 was added after IC, and elution was done with a solution containing 10% acetonitrile to guarantee sufficient solubility of the analyte in the final extract.

3.2. Internal standardization

A challenge of using IC for intact protein extraction is the selection of a proper internal standard (IS), which ideally should be added as early as possible in the analytical work-flow and, thus, correct for variability in a maximum number of steps: not only for fluctuations in chromatography and mass spectrometry, but also for differences in capturing efficiency by the antibody. The best choice for an IS in protein quantification is a stable-isotope labelled form of the analyte [11,17], but in practice such a compound is often difficult to obtain for researchers without access to the cell lines used to produce the recombinant protein of interest, or the associated costs may be prohibitive. For somatropin, a protein analogue containing six additional histidine moieties at the N-terminus is commercially available, and this compound was recognized by the anti-somatropin antibody and, therefore, extracted together with the analyte. The mass difference

Table 1
Overview of *m/z* values and mass extraction windows used for quantification of the different charge states of somatropin and internal standard.

| Compound	Charge state	Center extraction mass (*m/z*)	Mass extraction window (*m/z*)		
			1.0 Da	0.25 Da	0.0625 Da
Somatropin	14 +	1581.3000	1580.8000–1581.8000	1581.1750–1581.4250	1581.2688–1581.3313
Somatropin	15 +	1476.1000	1475.6000–1476.6000	1475.9750–1476.2250	1476.0688–1476.1313
Somatropin	16 +	1383.9000	1383.4000–1384.4000	1383.7750–1384.0250	1383.8688–1383.9313
Somatropin	17 +	1302.5000	1302.0000–1303.0000	1302.3750–1302.6250	1302.4688–1302.5313
Internal Standard	15 +	1530.1750	1529.6750–1529.9250		
Internal Standard	16 +	1434.6250	1434.1250–1434.3750		
Internal Standard	17 +	1350.2750	1349.7750–1350.0250		
Internal Standard	18 +	1275.3250	1274.8250–1275.0750		
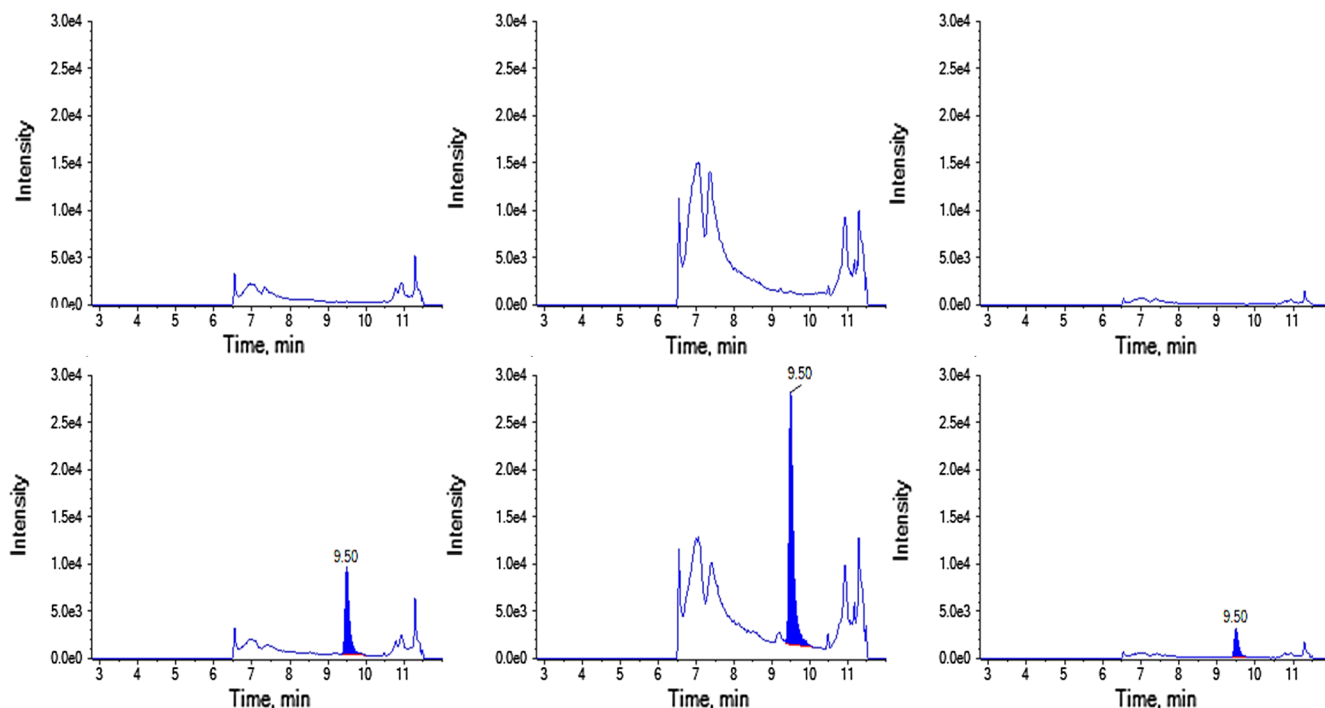
Fig. 1. Extracted ion chromatograms for intact somatropin; upper panels: blank rat plasma, lower panels: 10.0 ng/mL in rat plasma; quantification by: only 16^+ charge state with MEW 1.0 Da (left panels), 14^+ to 17^+ summed with MEW 1.0 Da (middle panels), 14^+ to 17^+ summed with MEW 0.0625 Da (right panels).
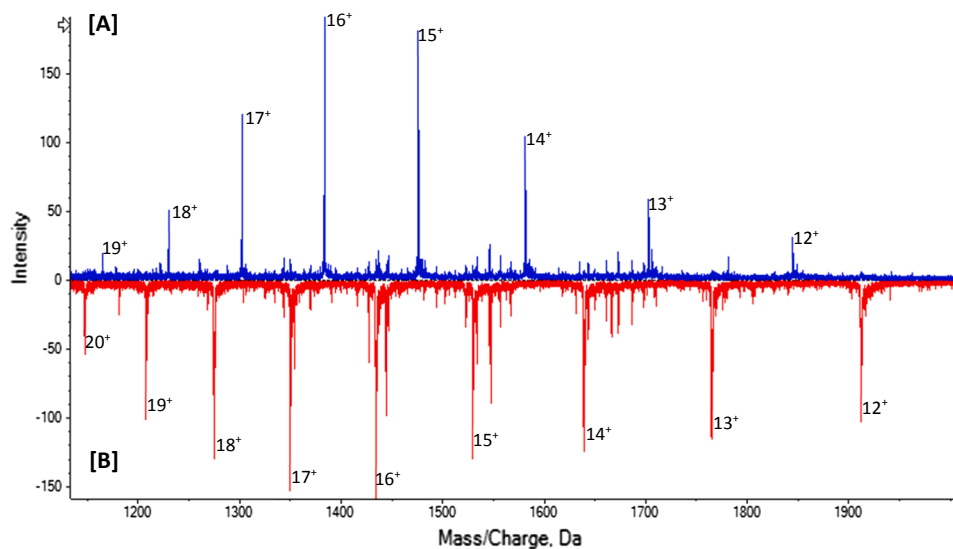

Fig. 2. Mass spectra for somatropin (A) and internal standard (B), showing their different charge states.

between analyte and internal standard was sufficient to distinguish them by mass spectrometry (see Fig. 2), so this internal standard could be readily used to correct for losses during sample preparation and other variability in the LC-MS assay.

3.3. Impact of in vivo protein binding

Another important aspect to consider is the potential interaction of a protein analyte with a macromolecular binding partner that occurs in the biological sample, such as the pharmacological target or a specific binding protein. Unbound and bound forms of the analyte exist in equilibrium *in vivo*, and while only the unbound fraction is initially available for extraction, removal of unbound analyte from the sample by the capture antibody may shift the equilibrium. In the most extreme case, the equilibrium is completely shifted and the total amount of

analyte extracted, but, depending on the differences in affinity of the two competing binding reactions, a lower recovery may also be obtained [18]. Since an extraction recovery of > 80% was found for spiked samples, the current method appears to extract something close the total concentration of somatropin. Equally importantly, unknown concentrations are calculated by reference to calibration samples which are assumed to display the same degree of *in vivo* protein binding, and thus also have a similar IC recovery. Therefore, concentrations found will always represent the total analyte fraction, even if the IC recovery is incomplete, as long as the matrix of calibrators and study samples is the same. To study the effect of potential differences in protein binding between samples of the same biological matrix, a recombinant form of the endogenous protein possibly involved in somatropin binding (soluble rat growth hormone binding protein) was added in a three-fold molar excess to a spiked rat plasma sample (500 ng/mL of somatropin)
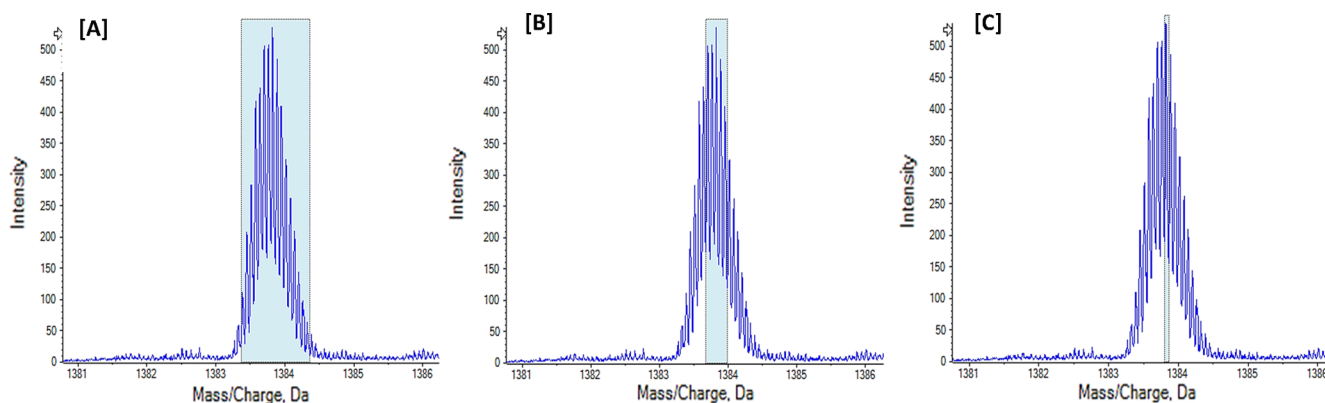
Fig. 3. Mass extraction windows of 1.0 Da (A), 0.25 Da (B) and 0.0625 Da (C) for the 16+ charge state of somatropin.

and incubated for 2 h at 37 °C. In this way, a study sample with an approximately three-fold higher than average concentration of the binding protein was generated, to represent a worst-case scenario. Quantification of this sample against a calibration curve without additional binding protein resulted in an accurate estimation of the added somatropin (bias: −5.8%, n = 3), which leads to the conclusion that sample-to-sample differences in binding protein levels do not impact the accuracy of the method and that total somatropin concentration are indeed obtained.

3.4. Selectivity of the immunocapture step

As with most commercial antibodies, the specific antigen-binding sites of the used anti-somatropin capture antibody are not known (or at least not disclosed), and therefore it is unknown which potential isoforms of somatropin the antibody actually recognizes. Very little is known about the *in vivo* fate of somatropin after dosing. In dose formulations, oxidized and deamidated forms of the protein have been identified [19] and these may potentially be formed *in vivo* as well. Since protein deamidation typically is a relatively slow process under physiological conditions, which occurs to a significant degree only after days to weeks [7], it is less likely to be relevant for a protein such as somatropin, with a half-life of just a few hours. We therefore focused on the oxidation of somatropin and prepared an oxidized form of the protein by treatment with hydrogen peroxide. Analysis by HRMS of a test solution obtained after 165 min of reaction, confirmed the presence of molecules with a mass increased by 16 and 32 amu, corresponding to the insertion of one or two oxygen atoms in the somatropin structure, at a relative abundance of 47% and 20%, respectively, and a low proportion (< 3%) of a compound with three oxygen atoms added (mass increase of 48 amu). Since somatropin contains three oxidizable methionines, it is likely that two of these are readily oxidized upon treatment with hydrogen peroxide and the third only to a much lesser extent, because of its location at the inside of the protein molecule making it less susceptible to possible modifications. Interestingly, these oxidized forms of somatropin – when added to plasma (1000 ng/mL) and subjected to the immunocapture step – were not recovered in the eluate at all. This suggests that the used capture antibody does not recognize these potential biotransformation products of somatropin, which would mean that the binding sites on the somatropin molecule, with this specific antibody, include one or more methionine moieties. It could also be that the protein denatures to some extent during the oxidation process and that the conformational epitopes necessary for immunocapture were lost. On the one hand, this finding shows that the IC step is highly selective, which is a positive feature if only concentrations of the dosed drug are required, but on the other hand it also demonstrates that these and other proteoforms may easily be missed upon IC extraction, which means a loss of potentially important information about the *in vivo* fate of a protein drug.

3.5. Chromatography and detection

Using an analytical LC column with a C4 stationary phase and a pore size of 300 Å, proper selectivity, retention and peak shape were found for intact somatropin and internal standard, provided that the column was kept at 80 °C, with lower temperatures resulting in peak broadening. No degradation products were formed at this relatively high chromatographic temperature (data not shown), demonstrating the suitability of the selected LC conditions. Operating at a linear gradient of acetonitrile increasing from 10% to 45% at 3.5% per minute, the protein analyte eluted at 9.5 min and the His-tag labelled internal standard eluted at 9.2 min. Including a step gradient at 85% acetonitrile and equilibration at the initial mobile phase composition of 10% acetonitrile, the total run time was 15 min per injection.

Upon positive-mode electrospray ionization, the intact somatropin molecule was converted into multiple ions with different *m/z* values, as shown in Fig. 2. Most abundant were the ions with 14, 15, 16 and 17 positive charges at *m/z* 1581.376, *m/z* 1476.008, *m/z* 1383.815 and 1302.483, respectively. For the internal standard, these were the ions with 15, 16, 17 and 18 positive charges at *m/z* 1530.175, *m/z* 1434.625, *m/z* 1350.275 and 1275.325, respectively. These ions each showed a further subdivision in about 20 partially resolved ions with different *m/z* values, which correspond to the masses of ions with increasing numbers of natural heavy isotopes, as exemplified in Fig. 3 for the 16-fold positively charged form of somatropin. Rather than selecting just one *m/z* value for detection, it is an option to summate the responses of several charge states and increase the total detection intensity. In addition, the so-called mass extraction window (MEW) can be increased around each of the selected *m/z* values to include more ions per charge state. Using these approaches, the total MS response will increase and, consequently, the detection sensitivity will improve, provided that background noise remains constant. The potential disadvantage is that, because a larger *m/z* range is included, matrix components with *m/z* values within this extended range will also generate responses that might interfere in the chromatograms.

To determine the effect of the summation of several charge states and of the width of the MEW on method performance, rat plasma samples spiked with somatropin at four levels (10.0, 30.0, 100 and 500 ng/mL) were analyzed and chromatograms recorded with different detection settings. Responses for somatropin were determined for each of the four most intense charge states (14+, 15+, 16+ and 17+) alone and for two, three and four charge states combined. In all cases, a MEW of 1.0 Da was used which corresponds to the inclusion of nearly all different isotopologues per charge state. For the situation in which the four most abundant charge states were summated, chromatograms were also recorded for MEW values reduced to 0.25 Da (corresponding to the four most intense ions of each charge state) and to 0.0625 Da (corresponding to only the most intense single ion per charge state). Example chromatograms recorded for somatropin in plasma at 10.0 ng/mL are

Table 2
Summary of accuracy and precision results (n = 18) obtained for four different detection settings.

Charge state(s)	MEW (Da)	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	CV (%)	Accuracy (%)
16	1.0	10.0	8.54	11.9	85.4
17 - 16 - 15 - 14	1.0	10.0	8.61	10.6	86.1
17 - 16 - 15 - 14	0.0625	10.0	8.70	10.6	87.0
Deconvoluted	N.a.	10.0	ND	–	–
16	1.0	30.0	27.6	13.4	92.1
17 - 16 - 15 - 14	1.0	30.0	26.9	13.1	89.7
17 - 16 - 15 - 14	0.0625	30.0	27.5	13.0	91.7
Deconvoluted	N.a.	30.0	26.9	12.0	89.8
16	1.0	100	103.9	7.0	103.9
17 - 16 - 15 - 14	1.0	100	102.9	7.2	102.9
17 - 16 - 15 - 14	0.0625	100	103.5	6.6	103.5
Deconvoluted	N.a.	100	107.0	11.2	107.0
16	1.0	500	524.2	14.4	104.8
17 - 16 - 15 - 14	1.0	500	529.7	14.0	105.9
17 - 16 - 15 - 14	0.0625	500	505.3	18.7	101.1
Deconvoluted	N.a.	500	531.8	8.1	106.4

ND: not detectable.
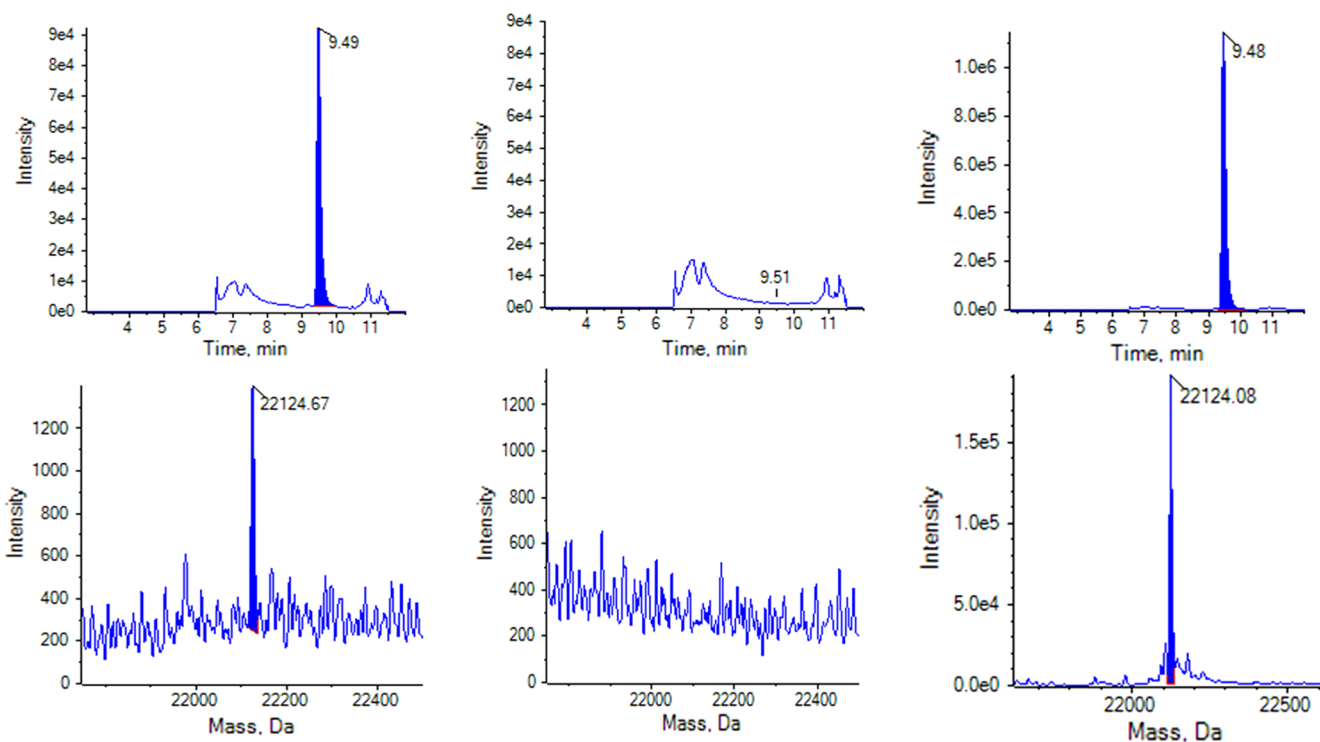
Fig. 4. Extracted ion chromatograms, 14^+ to 17^+ charge states summated with MEW 1.0 Da (upper panels) and deconvoluted mass spectra (lower panels) for extracted rat plasma samples: spiked plasma standard at 30.0 ng/mL (left panels), pre-dose samples (middle panels) and 0.5 h post-dose samples (right panels).

depicted in Fig. 1. Although the absolute detection response increases when more charge states are included and when the MEW is larger, the signal to noise ratio in the chromatograms is essentially unaffected, which indicates that along with more analyte signal more background noise is also extracted. Furthermore, there is no clear indication of the appearance of additional peaks in the chromatograms when the number of charge states or the MEW is increased. This means that no endogenous matrix components are present in the plasma extracts with m/z values falling in the ranges used for the detection of somatropin.

3.6. Method performance

All spiked samples were analyzed in six-fold on three separate days. Results were calculated against a spiked calibration curve in rat plasma, which was analyzed using the same settings, and precision and accuracy were determined. An overview of the results is included in Table S-2 in the supplementary material with a summary shown in Table 2. For all detection settings, the values for precision (expressed as coefficient of variation) were below 15%, except for the highest concentration that was measured with a reduced MEW, where it was between 15% and 20%. Values for accuracy (average result found relative to the nominal spiked concentration) were between 85% and 115%. These results show that method performance in terms of selectivity, obtainable concentration sensitivity, accuracy and precision is essentially independent of the MEW and the number of charge states used. This is unlike what we found earlier for the signature peptides of digested somatropin [14], where interference in the chromatograms was greatly reduced and accuracy and precision were much improved by narrowing the MEW.
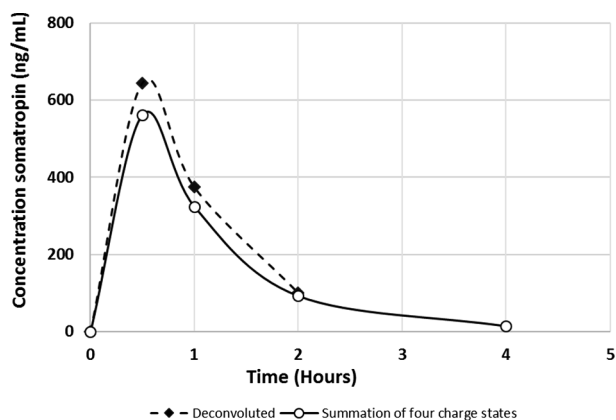
Fig. 5. Intact somatropin concentrations in rat plasma after a single 2-mg/kg subcutaneous dose.

Very probably, this is due to the extensive clean-up of the plasma sample by the immunocapture step and the virtual absence of interfering compounds in the extracts in the current method, while the direct analysis of a plasma digest in the digestion-based method leads to generation of a large number of interfering peptides and their subsequent introduction into the LC-MS system. Altogether, the use of a MEW of 1.0 Da gives acceptable results. It could therefore be speculated that analysis of intact somatropin on a low-resolution mass spectrometer, such as a triple quadrupole or ion-trap, may be a possibility, if such a system allows measurement of ions with *m/z* values up to about 2000 and as long as samples are pretreated by immunocapture.

Deconvolution of mass spectra leads to reconstructed spectra showing the calculated mass of the neutral protein (see Fig. 4). The ratio of the area of this deconvoluted neutral mass peak (22,124 Da) to that of the corresponding internal standard was used as the analytical response for each of the samples. The results for accuracy and precision are included in Table 2 and show that the performance of the deconvolution approach is comparable to using a limited number of charge states. The detection sensitivity of the deconvolution approach, however, is less favorable than that of using EICs (Fig. 4). For the current method, the sample at 10 ng/mL was undetectable but the 30-ng/mL level showed acceptable performance in terms of precision and

accuracy (CV and bias < 15%) and this level was therefore selected as a workable LLOQ with the deconvolution approach.

3.7. Analysis of preclinical samples

Based on the results described above, it was decided to select the summation of the four most intense charge states, extracted with a MEW of 1.0 Da, as the final detection setting for further analysis. Fig. 5 shows an example of a pharmacokinetic curve as obtained for intact somatropin after subcutaneous 2 mg/kg dosing to a rat, by immunocapture and LC–HRMS analysis. The figure demonstrates that the LLOQ of 10 ng/ml is sufficient to monitor the relevant somatropin concentrations in rat plasma after this dose up to 4 h post-dose. The results obtained in the same samples by deconvolution of the detection signals are included in Fig. 5. The latest time-point is unquantifiable because of the higher LLOQ after deconvolution, but otherwise the data of both quantification approaches agree quite well. When comparing the concentration data (obtained after analysis of all rat plasma samples) of the EIC approach to those of the deconvolution approach, a good correlation ($R^2 > 0.97$) was found. On average, the results after summation of the four most abundant ions were 12% lower than when using deconvolution (Fig. 6). This is somewhat surprising, because the concentrations in the preclinical samples were calculated against calibrators whose raw data were processed in the same way. Therefore, it would imply that a systematically higher response is found in the preclinical samples than in the corresponding spiked calibration samples with the deconvolution approach but not with the EIC approach. The spiked validation samples did not show this discrepancy (see: Table 2), so the reason for this (slight) deviation remains unclear.

4. Conclusion

LC-HRMS can be successfully used for the quantitation of intact proteins down to the low ng/mL level in a complex, protein-rich biological matrix, when used in combination with immunocapture extraction as a selective sample preparation step. For the 22-kDa protein somatropin, an LLOQ of 10 ng/mL (0.45 nM) was obtained in 75 μL of rat plasma, using a Q-TOF mass spectrometer with a mass resolution of about 40,000. The summation of the responses of the four most abundant charge states and the use of a relatively large mass-extraction window of 1.0 Da increased the absolute detection sensitivity but did
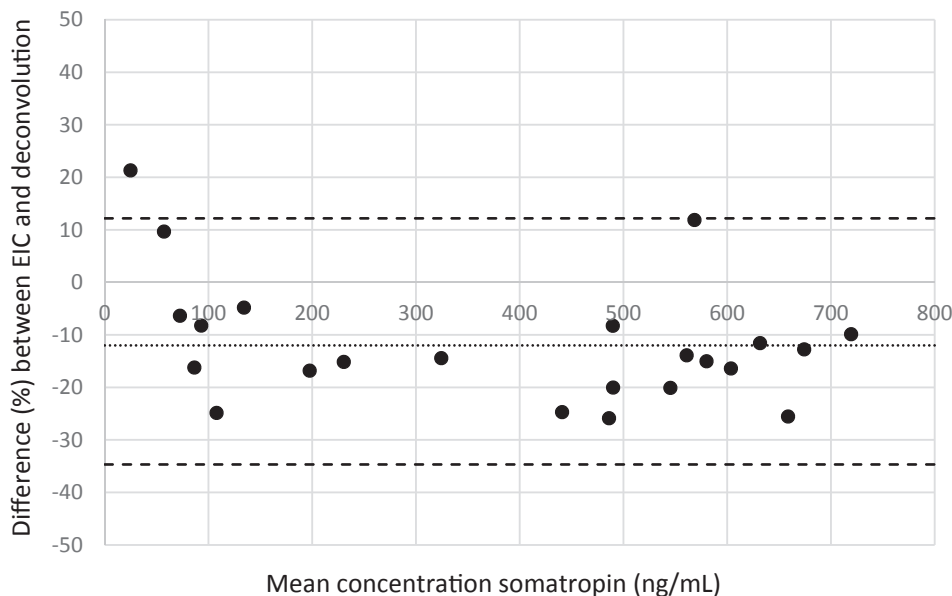

Fig. 6. Comparison of somatropin concentrations (n = 23) found in rat plasma after a single 2-mg/kg subcutaneous dose: Bland-Altman plot with mean and 95% limits of agreement (mean bias (%) ± 1.96 standard deviation).

not substantially improve the LLOQ because the signal to noise ratio remained the same. Deconvolution of the mass spectra and using the resulting processed data for quantification gave equally acceptable results, although the obtainable LLOQ was less favorable at 30 ng/mL. A previously published LC-MS/MS method for somatropin, which involved digestion of the plasma sample and quantification of three surrogate peptides, had an LLOQ of 25 ng/mL [12]. This shows that intact protein quantification by LC-HRMS has the potential of better sensitivity, if it is used in combination with a thorough sample clean-up by immunocapture extraction. Here, it should be realized that somatropin is a relatively small protein with rather straightforward chromatographic and mass spectrometric properties. It is structurally less complex than monoclonal antibodies, the most widely used class of biopharmaceuticals, for which other conclusions have been drawn [13]. The effect of a potentially variable binding of somatropin to endogenous plasma proteins on immunocapture recovery was found to be negligible. Although a good protein internal standard was commercially available for this particular analyte, finding an adequate internal standard remains a potential challenge for intact protein analysis in general.

CRediT authorship contribution statement

Peter Bults: Conceptualization, Methodology, Validation, Investigation, Writing - original draft. **Anders Sonesson:** Resources, Writing - review & editing. **Magnus Knutsson:** Resources, Writing - review & editing. **Rainer Bischoff:** Supervision, Writing - review & editing. **Nico C. van de Merbel:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2020.122079.

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