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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 LFDNAMLRL with a $^{13}\text{C}_6$ - $^{15}\text{N}_4$ -labeled C-terminal arginine and FDTNSHNDDALLK with a $^{13}\text{C}_6$ - $^{15}\text{N}_2$ -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 somatotropin.

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
LFDNAMLRL	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
LFDNAMLRL [‡]	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 ¹³C₆¹⁵N₂-labeled internal standard.

[‡]C-terminal arginine ¹³C₆¹⁵N₄-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 K₃-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 LFDNAMLRL (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 LFDNAMLRL 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 LFDNAMLRL, 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 LFDNAMLRL 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^{+1})) 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 $\pm 20\%$ 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 LFDNAMLRL 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 (%)
FDTNSHNDALLK	497.2	614.3090 (y ₁₁) + 671.8236 (y ₁₂)	0.07	25	15.7	106.6
				50	10.6	103.7
				75	4.4	110.1
				1250	5.4	95.1
				8000	6.4	88.9
SNLELLR	422.8	401.2871 (y ₃) + 530.3328 (y ₄)	0.01	25	11.9	103.3
				50	10.4	98.7
				75	10.9	99.6
				1250	9.0	97.3
				8000	6.8	91.7
LFDNAMLRL	490.3	604.3271 (y ₅) + 719.3563 (y ₆) + 866.4330 (y ₇)	0.01	25	11.3	88.6
				50	16.3	80.8
				75	7.2	110.2
				1250	12.7	105.4
				8000	1.8	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 y₁₁ and y₁₂ gave the best results. For peptide LFDNAMLRL, summation of three product ions y₅, y₆ and y₇ 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
LFDNAMLRL	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 LFDNAMLRL (□) 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 LFDNAMLRL 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.

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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.

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.

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