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Comparison of Triple Quadrupole and Orbitrap Mass Spectrometry for Quantitative Bioanalysis of Intact Proteins

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Abstract

Background: Ligand binding assays (LBA) are still the golden standard for the quantitative analysis of therapeutic proteins and protein biomarkers in biological matrices. Alternatively, bioanalytical methods for proteins have been developed using liquid chromatography and mass spectrometry (LC-MS) after enzymatic digestion (bottom-up approach). Quantitative LC-MS analysis of intact proteins (MW>5 kDa) for bioanalytical purposes is still in its infant stage and needs to be explored.

Methods: To investigate the potential limit of detection (LOD) and calibration range for small and large intact proteins, four proteins were selected, i.e. Cytochrome C (12.4 kDa), Human Serum Albumin (66.5 kDa), apo-Transferrin (79.5 kDa) and Infliximab (Remicade®, 148.5 kDa). The experiments were performed on two state-of-the-art UPLC-MS systems: a triple quadrupole mass spectrometer (XEVO-TQS) and a high-resolution orbitrap mass spectrometer (Q-Exactive).

Results: The triple quadrupole mass spectrometer enabled quantitative analysis of intact proteins at LOD levels ranging from approximately 10 to 100 ng/ml (MRM mode) whereas the LOD obtained with the high-resolution orbitrap mass spectrometer was in the range 50-500 ng/ml. The calibration curves were non-linear and the calibration range was limited, i.e. approximately two orders of magnitude.

Conclusion: LBA, LC-MS (bottom-up) and LC-MS (top-down) are complementary techniques. The LC-MS (top-down) approach has added value if LBA or LC-MS (bottom-up) do not offer the desired result. For the quantitative LC-MS analysis of intact proteins in a complex biological matrix selective sample pre-treatment is required.

Keywords: Intact proteins; Mass Spectrometry; Quantitative Analysis; Bioanalysis

Introduction

Ligand binding assays (LBA) are still the golden standard for the quantitative analysis of therapeutic proteins and protein biomarkers in biological matrices. LBA are the most sensitive assays for the quantification of proteins in biological matrices, and they enable high-throughput bioanalysis. However, assay development is rather time consuming, the dynamic range is limited and selectivity issues are not unusual in LBA. Alternatively, bioanalytical methods for therapeutic proteins have been developed using liquid chromatography and mass spectrometry (LC-MS) after enzymatic digestion (bottom up approach). Many papers have been published about the enzymatic digestion procedures, selection of appropriate surrogate peptides, sample clean-up and the use of internal standards [1,2]. Potential advantages of the mass spectrometric approach are the faster method development time, the increased linear dynamic range, higher selectivity and the easy-to-implement multiplex methods. Neither LBA nor the LC-MS approach offers the ultimate solution to the complex analytical challenges [3]. Depending on the underlying analytical principle, the use of the different methodologies can lead to very different concentration results for the same sample [3]. However, Liu and coworkers [4] showed that PK profiles using data generated from two different platforms (LBA and bottom-up LC-MS) matched very well.

Intact proteins at relatively high concentrations in aqueous solutions are often characterized using MALDI-TOF, (Q)TOF- or Orbitrap mass spectrometers. In addition, a relatively new field, termed native protein mass spectrometry, is

an expanding approach for studying intact biomolecular structure in the near-native state, especially proteins and protein complexes, in the gas phase [5]. However, for quantitative and bioanalytical purposes, the mass spectrometric analysis of intact proteins (MW>5 kDa) is still in its infant stage [6]. To extract intact proteins from a biological matrix solid-phase extraction (SPE), immuno-depletion and immuno-affinity extraction have been described. SPE has been used mainly to extract smaller proteins up to 14.3 kDa (Lysozyme) from a biological matrix [7-9]. Solid phase sorbents with strong anion exchange (SAX), non-polar (reversed phase, C8), polar and mixed properties were reported.

Immuno-depletion is applied to capture the most abundant plasma proteins, whereas the protein of interest elutes without retention. Six commercially available immuno-depletion products were compared by Polaskova et al [10]. Immuno-depletion is not the method of choice if high sensitivity is required, i.e. only a few proteins are depleted. All other endogenous compounds are still present in the plasma sample [11], and may influence source ionization, chromatographic performance during LC-MS analysis and/or the specificity of the method.

Immuno-affinity extraction is the most selective sample pre-treatment technique [12-17]. The technique consists of the following steps: (I) an antibody raised against the protein analyte is bound to a solid support (column format or beads), (II) the plasma sample containing the protein analyte is mixed with the bound antibody, (III) all non-specific bound plasma constituents are washed away and (IV) the protein analyte is eluted from the antibody. Captured proteins can be eluted with a small volume of water or buffer (usually low pH) with or without organic modifier while shaking/mixing for 15-60 min. Finally, the very clean extract is analyzed with LC-MS. The extracts obtained after immuno-affinity extraction enable LC-MS analysis with hardly any matrix effects.

Intact proteins are separated by reversed-phase chromatography using hydrophobic stationary phases [7-9,11-18,20]. Silica-based stationary phases with a hydrophobic octadecyl group (C18) or shorter hydrophobic groups (C4, C5, C8), as well as polymeric stationary phases have been reported (particle size from 1.7 µm up to 10 µm). However, ion-exchange and size-exclusion columns are also regularly described [21-22]. Pore sizes of 80-100 Å (smaller proteins) up to 2000-4000 Å (largest proteins) have been used. Use of a polymer monolith column with large and small pores was reported by Gafvels et al. [14]. Most researchers have used narrow-bore (± 2 mm ID) LC columns with column lengths ranging from 10 mm to 150 mm. Capillary LC (0.3 mm ID) was reported for the separation of an Antibody Drug Conjugate (ADC) to determine the drug-antibody ratio (DAR) distribution [17]. The applied LC column temperature was 30-50°C (smaller proteins) up to 70-75°C (larger proteins). The mobile phases for gradient elution consisted of water acidified with formic acid or acetic acid and an organic solvent (acetonitrile or methanol). Use of a low percentage (0.02-0.05%) trifluoroacetic acid (TFA) as ion-pairing agent was reported to improve the chromatographic peak shape of the protein.

Intact proteins with masses up to approximately 150 kDa have been quantified in biological matrices using LC-MS. Triple quadrupole, ion trap [13] and high-resolution MS instruments, such as orbitrap MS [9,14] and Time of Flight (TOF) MS [17-18], have been reported for the (quantitative) bioanalysis of intact proteins. All proteins were ionized with electrospray ionization in the positive mode, leading to the typical envelope of multiply charged ions. Most intact proteins were analyzed without internal standard. However, a structural analogue or a stable-isotope labelled protein was used for some applications [7,9]. On triple quadrupole instruments the limit of quantification (LOQ) varied from 0.5 ng/ml (EPI-hNE4, 6.2 kDa) [12] up to 1 µg/ml (trastuzumab, 150 kDa) [19]. High resolution mass spectrometers are generally operated in the full scan acquisition mode. The very high resolution may affect the sensitivity of the method. Summation of extracted ion chromatograms improved the LOQ of the method. For Lyzosyme (14.3 Da), an LOQ of 0.1 µg/ml [18] and 0.5 µg/ml [9] was reported. High resolution mass spectrometers have also been used for "qualitative" bioanalysis, such as characterization of post-translational modifications [9], measurement of ApoA-I proteoforms [14], Transferrin isoforms ([15], characterization of human serum albumin dimer [20] and determination of the DAR distribution of an ADC [17].

Based on the published papers about LC-MS analysis of intact proteins in biological matrices the information about the quantitative possibilities and the potential limit of detection for (especially larger) intact proteins is still limited. Therefore, this paper focuses on the quantitative (bio) analysis of small and large intact proteins using two state-of-the-art UPLC-MS systems: a triple quadrupole mass spectrometer (XEVO-TQS) and a high-resolution orbitrap mass spectrometer (Q-Exactive). For the investigation four proteins were selected, i.e. Cytochrome C (12.4 kDa), Human Serum Albumin (66.5 kDa), apo-Transferrin (79.5 kDa) and Infliximab (Remicade®, 148.5 kDa). The aim of the experiments was to determine the potential limit of detection and the calibration range for each protein. After method development, intact proteins were analyzed in a biological matrix.

Experimental

Chemicals

Cytochrome C (from equine heart; C2506), Albumin (from human serum, HSA, A3782), apo-Transferrin (bovine, T1428), L-Arginine (A006), L-Glutamic acid (G6904), sodium chloride, Maltotriose hydrate (95%), Tween 80 (BioXtra Quality), Trifluoroacetic acid (TFA, Chromasolv quality >99%) and sodium acetate were obtained from Sigma Aldrich (St Louis, MO, USA). Infliximab (Remicade®, 10 mg/ml) was kindly provided by the University Medical Centre

(Utrecht, The Netherlands). Acetic acid was purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN, ULC/MS – CC/SFC Quality), isopropanol (IPA, ULC/MS Quality) and formic acid (FA, ULC/MS Quality 99%) were supplied by Biosolve (Valkenswaard, The Netherlands). m-Nitrobenzyl alcohol (m-NBA, MS Quality >99.5%) was ordered from Fluka (Sigma Aldrich). Water was purified in-house (Millipore: MilliQ quality >18.2 Mohm).

Calibration curves

Stock solutions of the selected proteins (Cytochrome C, HSA, Apo-transferrin) were prepared at a concentration of 10 mg/ml in a solution consisting of L-arginine (50 mM), L-glutamic acid (50 mM), sodium chloride (100 mM) and sodium acetate buffer (20 mM) in water, adjusted to pH 5.3 with acetic acid. Calibration standards were prepared at concentration levels of approximately 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 µg/ml in Tween 80/maltotriose (0.005%/0.5%) in water. After analysis of the calibration standards the integrated MS response of individual proteins (y-axis) was plotted versus the actual protein concentration (x-axis). Quadratic regression analysis with weighting factor $1/x^2$ was applied. The limit of detection (LOD) was determined as the lowest protein concentration in a calibration standard which can be quantified reliably, with an acceptable accuracy and precision. In addition, the protein signal of the LOD sample should be at least 3 times the signal of a blank sample (Tween 80/maltotriose (0.005%/0.5%) in water).

Bioanalytical experiments

Mouse serum (Strain BALB/cOlaHsd; female; batch HNL131107F), obtained from Harlan (The Netherlands), was pre-treated using ultrafiltration (Centrifugal Filters Amicon®Ultra-0.5 ml, Ultracel®-10K, Regenerated Cellulose 10,000 NMWL, UFC 501096, Merck Millipore Ltd, Ireland). The serum ultrafiltrate was spiked with protein at concentration levels of approximately 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 µg/ml. The spiked ultrafiltrates were analyzed using UPLC-MS (XEVO-TQS and Q-Exactive). The LOD was determined as the lowest protein concentration in mouse serum ultrafiltrate which can be quantified reliably, with an acceptable accuracy and precision. In addition, the protein signal of the LOD sample should be at least 3 times the signal of a blank sample (blank mouse serum ultrafiltrate).

UPLC-MS equipment and conditions (QqQ)

Cytochrome C, HSA, apo-Transferrin and Infliximab were analyzed using UPLC (Acquity, Waters) and a XEVO-TQS mass spectrometer (Waters). An Acquity BEH300 C4, 2.1 × 100, 1.7 µm, 300 Å column (Waters) was used at a temperature of 75°C. The autosampler temperature was 15°C. The injection volume was 7.5 µl. All proteins were analyzed using electrospray ionization in the positive mode (z-spray). Full scan, selected ion monitoring (SIM) as well as multiple reaction monitoring (MRM) procedures were set up. The total ion current (TIC) of all signals obtained from the SIM and MRM procedures was used for quantification to obtain a better signal to noise ratio. Masslynx software (version 4.1, Waters) was used for data acquisition, integration and processing.

Cytochrome C (QqQ): Mobile phase A and mobile phase B consisted of 0.1% FA in water and 0.1% FA in ACN, respectively. The UPLC gradient is shown in Table 1. The electrospray voltage was 1.25 kV. Other settings were: Desolvation temperature (600°C), Cone gas flow (150 l/Hr) and Desolvation gas flow (1200 l/Hr). For SIM, the TIC of m/z 687.6, m/z 728.0, m/z 773.6 and m/z 883.9 was used for quantification. For MRM, the immonium-fragments 120.0 and 136.0 m/z were monitored for each of the parent ions 687.6, 728.0, 773.6, 825.0 and 883.9 m/z. The dwell time and cone voltage were 25 ms and 50 V for all transitions. The collision energy was 70 eV (daughter ion m/z 120.0) and 50 eV (daughter ion m/z 136.0). The TIC of all MRM transitions was used for quantification to increase the sensitivity.

HSA and apo-Transferrin (QqQ): Mobile phase A consisted of water/IPA (98/2, V/V) with TFA (0.1%). Mobile phase B consisted of IPA/ACN/water (70/20/10, V/V/V) with TFA (0.1%). The UPLC gradient is shown in Table 2. The spray voltage was 3.5 kV. Other settings were: Desolvation temperature (600°C), Cone gas flow (150 l/Hr) and Desolvation gas flow (1200 l/Hr). The dwell time, the cone voltage and the

Time (min)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
Initial	0.5	99.0	1.0
6.50	0.5	50.0	50.0
8.00	0.5	10.0	90.0
8.50	0.5	10.0	90.0
8.60	0.5	99.0	1.0
10.00	0.5	99.0	1.0

Table 1: UPLC gradient for analysis of Cytochrome C.

Time(min)	Flow rate(ml/min)	Mobile phase A(%)	Mobile phase B(%)
Initial	0.5	80.0	20.0
6.50	0.5	50.0	50.0
8.00	0.5	10.0	90.0
8.50	0.5	10.0	90.0
8.60	0.5	80.0	20.0
10.00	0.5	80.0	20.0

Table 2: UPLC gradient for analysis of HSA, apo-Transferrin and Infliximab.

collision energy were 25 ms, 50 V and 120 eV, respectively, for all ten transitions. The TIC of all MRM transitions was used for quantification.

MS conditions for HSA (QqQ): For SIM, the TIC of m/z 1387.2, m/z 1448.1, m/z 1550.3, m/z 1586.9, m/z 1624.2, m/z 1664.3, m/z 1754.1 and m/z 1958.1 was used to increase the signal to noise ratio. For MRM, the immonium-fragments m/z 70.0 and m/z 84.0 were monitored for each of the parent ions m/z 1387.2 , m/z 1448.1 ,m/z 1586.9, m/z 1624.2 and m/z 1664.3.

MS conditions for apo-Transferrin (QqQ): For SIM, the TIC of m/z 1530.2, m/z 1592.0, m/z 1626.0, m/z 1659.7, m/z 1733.6, m/z 1773.2, m/z 1815.9 and m/z 1955.8 was used to increase the signal to noise ratio. For MRM, the immonium-fragments m/z 70. 0 and m/z 84. 0 were monitored for each of the parent ions m/z 1592.0, m/z 1626.0, m/z 1659.7, m/z 1773.2 and m/z 1815.9.

Infliximab (QqQ): Mobile phase A consisted of water/IPA (98/2, V/V) with m-NBA (0.5%) and TFA (0.1%). Mobile phase B consisted of IPA/ACN/water (70/20/10, V/V/V) with m-NBA (0.5%) and TFA (0.1%). The applied gradient is shown in Table 2. The spray voltage was 3.5 kV. Other settings were: Desolvation temperature (600°C), Cone gas flow (150 l/Hr) and Desolvation gas flow (1200 l/Hr). For full scan MS, data were acquired in the range from m/z 1400 to m/z 2000 (cone voltage 80 V, scan time 0.3 s). For MRM, the immonium-fragments m/z 70.0 and m/z 84.0 were monitored for each of the parent ions m/z 1633.7, m/z 1692.2, m/z 1733.0, m/z 1792.5 and m/z 1839.4. The dwell time, the cone voltage and the collision energy were 25 ms, 80 V and 120 eV, respectively, for all ten transitions. The TIC of all MRM transitions was used for quantification.

UPLC-MS equipment and conditions (HRMS)

HSA, apo-Transferrin and Infliximab were analyzed using UPLC (Dionex Ultimate 3000) and a Q-Exactive mass spectrometer (ThermoElectron). An Acquity BEH300 C4, 2.1 × 100, 1.7 µm, 300 Å column (Waters) was used at a temperature of 75°C. Mobile phase A consisted of water/IPA (98/2, V/V) with TFA (0.1%). Mobile phase B consisted of IPA/ACN/water (70/20/10, V/V/V) with TFA (0.1%). The applied gradient is shown in Table 2. The autosampler temperature was 15°C. The injection volume was 7.5 µl. All proteins were analyzed using electrospray ionization in the positive mode (HESI source). The spray voltage was 6.0 kV. Other settings were: Sheath gas flow (60 AU), Auxiliary gas flow (20 AU), capillary temperature (350°C), heater temperature (350°C) and S-Lens RF Level (80 V). The resolution was 17,500. Other MS settings were: In-source CID (80 eV), AGC target ($3e^6$), Maximum IT (100 ms) and microscans (2). XCalibur software version 2.2 (ThermoScientific) was used for data acquisition, integration and processing.

HRMS conditions for HSA: The scan range was m/z 1200 to m/z 3200. The signals obtained at m/z 1958.4810, m/z 2027.6230, m/z 2080.8480, m/z 2147.9190, m/z 2219.503, m/z 2296.085, m/z 2378.025, m/z 2466.093, m/z 2560.809, m/z 2663.264, m/z 2774.065, m/z 2895.253, m/z 3026.398 and m/z 3170.260 with a mass window of 500 ppm were summed to obtain the best sensitivity.

HRMS conditions for apo-Transferrin: The scan range was m/z 1500 to m/z 3500. The signals obtained at m/z 2037.223, m/z 2044.286, m/z 2092.20294, m/z 2099.589, m/z 2158.420, m/z 2166.6845, m/z 2220.2158, m/z 2228.857, m/z 2284.8542, m/z 2294.343, m/z 2353.8595, m/z 2364.704, m/z 2427.6847, m/z 2437.61290, m/z 2505.8003, m/z 2515.605, m/z 2589.8884, m/z 2600.863, m/z 2678.9486, m/z 2689.129, m/z 2774.288, m/z 2784.3791 and m/z 2888.333 with a mass window of 500 ppm were summed to obtain the best sensitivity.

HRMS conditions for Infliximab: The scan range was m/z 2000 to m/z 6000. The signals obtained at m/z 3710-3730, m/z 3805-3825, m/z 3905-3925, m/z 4005-4025, m/z 4235-4255, m/z 4360-4380, m/z 4490-4510, m/z 4640-4660, m/z 4790-4810 were summed to obtain the best sensitivity.

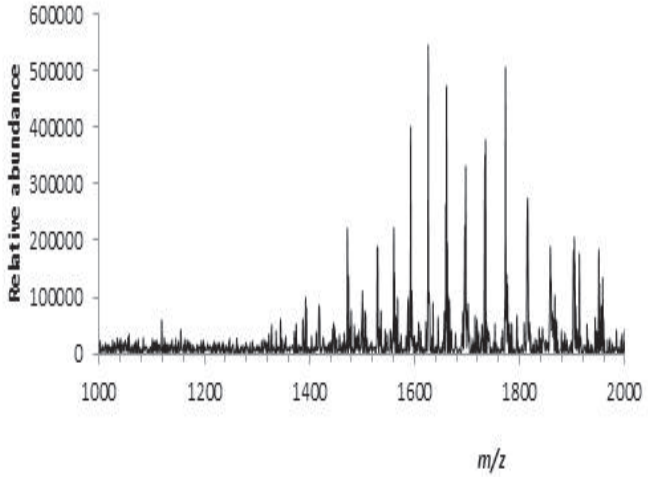

Figure 1: Mass spectrum of Apo-Transferrin in ACN/water (15/85, V/V) with TFA (0.1%) using a triple quadrupole mass spectrometer (XEVO-TQS)

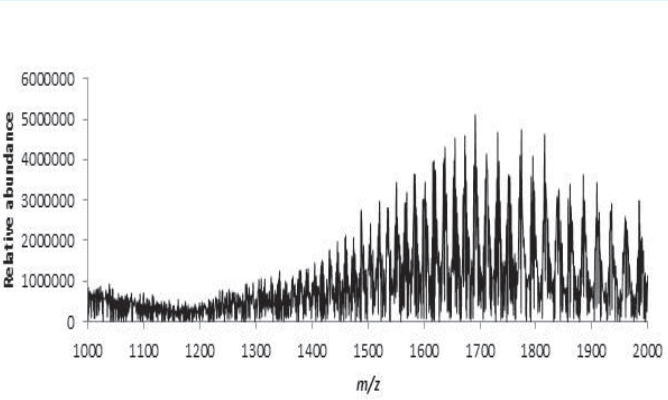

Figure 2: Mass spectrum of Infliximab in water/IPA (98/2, V/V) with m-NBA (0.5%) and TFA (0.1%) using a triple quadrupole mass spectrometer (XEVO-TQS).

Protein	Molecular weight (kDa)	XEVO-TQS SIM Calibration range µg/ml	XEVO-TQS SIM LOD (µg/ml)
Cytochrome C	12.4	0.20-10	0.20
HSA	66.5	0.50-10	0.50
apo-Transferrin	79.5	0.20-10	0.20
Infliximab*	148.5	0.20-10	0.20
*Full scan (m/z 1400-2000).			

Table 3: Calibration range and Limit of Detection (LOD) of proteins analysed with UPLC and a triple quadrupole mass spectrometer (XEVO-TQS). The calibration standards were prepared in Tween 80/ maltotriose (0.005%/0.5%) in water. The proteins were analysed in Selected Ion Monitoring **(SIM)** mode.

Protein	Molecular weight(kDa)	XEVO-TQS MRM Calibration range (µg/ml)	XEVO-TQS MRM LOD (µg/ml)
Cytochrome C	12.4	0.010-1.0	0.010
		(0.10-10)	
HSA	66.5	0.10-10	0.10
apo-Transferrin	79.5	0.050-10	0.050
Infliximab	148.5	0.050-1.0	0.050
		(0.20-10)	

Table 4: Calibration range and LOD of proteins analysed with UPLC and a triple quadrupole mass spectrometer (XEVO-TQS). The calibration standards were prepared in Tween 80/maltotriose (0.005%/0.5%) in water. The proteins were analysed in Multiple Reaction Monitoring **(MRM)** mode.

Results

Optimization of UPLC conditions for intact proteins

The UPLC conditions were optimized for the analysis of the selected proteins Cytochrome C, HSA, apo-Transferrin and Infliximab with molecular weights ranging from 12.4 up to 148.5 kDa. An Acquity BEH300 C4 column was used at a column temperature of 75°C. The mobile phase composition was optimized for each protein. TFA was added to the mobile phase for the analysis of the larger proteins to improve the peak shape. After optimization, acceptable peak shapes were obtained but at concentrations below approximately 1 µg/ml, the analysis of intact proteins was not successful due to severe loss of proteins which was presumably caused by adsorption to materials. To minimize adsorption all protein solutions were prepared in plastic vials and a mixture of Tween 80/Maltotriose (0.005%/0.5%) was added to the protein solutions [23-24].

UPLC-MS of intact proteins using a triple quadrupole mass spectrometer

Method development was initiated by the acquisition of mass spectra of the 4 proteins. The mass spectrum of apo-Transferrin is presented in Figure1. Apo-Transferrin, a 79.5-kDa protein, could be measured in water/ACN/TFA

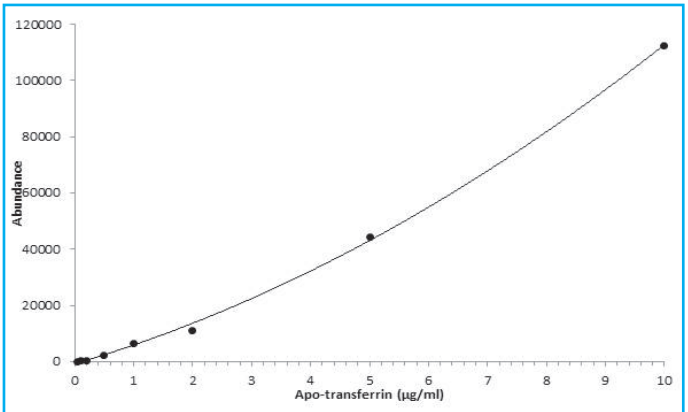

Figure 3: Calibration curve of Apo-Transferrin in mouse serum ultrafiltrate (XEVO-TQS, MRM). The calibration range was from 0.05 µg/ml to 10 µg/ml. Quadratic regression analysis with weighting factor $1/x^2$ was applied.

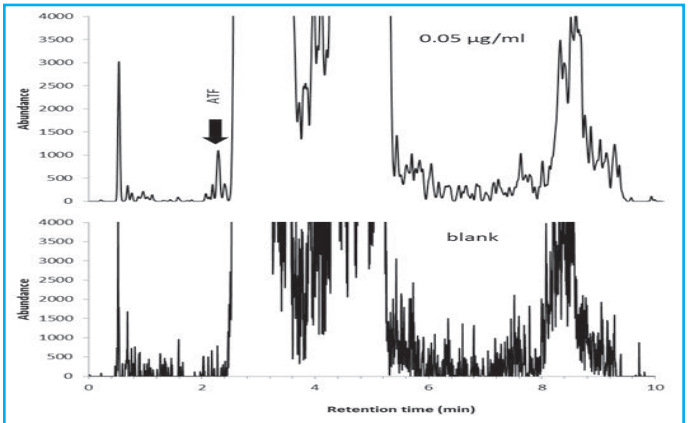

Figure 4: UPLC-MS/MS chromatogram of Apo-Transferrin (0.05 µg/ml) in mouse serum ultrafiltrate (upper chromatogram) and blank mouse serum ultrafiltrate (lower chromatogram). The samples were analyzed using a triple quadrupole mass spectrometer (XEVO-TQS, MRM).

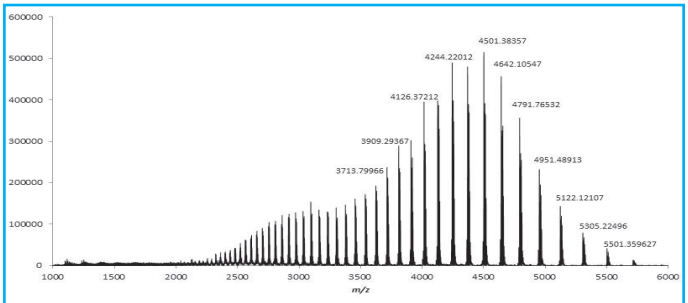

Figure 5: Mass spectrum of Infliximab in ACN/water (15/85, V/V) with TFA (0.1%) using a high resolution orbitrap mass spectrometer (Q-Exactive).

(85/15/0.1, V/V) within the mass range of the XEVO-TQS mass spectrometer (m/z range up to 2000) due to the multiple charging caused by the electrospray ionization. The most predominant ions were observed in the range m/z 1530.2 [M+52H$^+$] to m/z 1955.8 [M+41H$^+$]. Cytochrome C and HSA could also be measured within the mass range of the mass spectrometer. However, intact Infliximab could not be analyzed using conventional mobile phases containing only FA or TFA, although heavy and light chains could be detected separately. By adding 0.5% (V/V) m-nitrobenzyl alcohol to the mobile phase [25] the charge state of Infliximab was increased which enabled the analysis of intact Infliximab within the mass range of the triple quadrupole mass spectrometer (Figure 2).

Using the acquired mass spectra selected ion monitoring (SIM) and multiple reaction monitoring (MRM) methods were set up for the four proteins. The total ion current (TIC) of all signals obtained from the SIM and MRM procedures was used for the quantification to obtain a better signal to noise ratio. The parent ions of Cytochrome C fragmented to immonium-ions 120.0 m/z (phenylalanine) and 136.0 m/z (tyrosine). For the larger proteins (HSA, apo-Transferrin and Infliximab) the parent ions typically fragmented to daughter ions with m/z 70.0 (arginine, asparagine, aspartic acid, lysine, proline) and m/z 84.0 (glutamine, lysine).

Calibration standards, prepared at protein concentrations ranging from 0.01 µg/ml to 10 µg/ml in Tween 80/ maltotriose (0.005%/0.5%) in water, were analyzed both in SIM and MRM mode (full scan for Infliximab). The results are presented in Table 3 (SIM) and Table 4 (MRM). The calibration range in the tables indicates the range in which the accuracy was within the acceptance criterion of ≤15% (≤20% at lowest level). All calibration curves showed non-linear behavior, possibly caused by adsorption of the proteins in the low calibration range, despite the addition of Tween/ maltotriose to reduce adsorption to surfaces, or ionization saturation in the high calibration range. As a consequence, all calibration curves were fitted using quadratic regression analysis with weighting factor $1/x^2$. The calibration range was relatively short for most proteins, i.e. approximately two orders of magnitude. For some proteins two calibration ranges have been reported, one at lower levels and one at higher levels. As clearly demonstrated in Table 3 and 4, the Limit of Detection (LOD) was better for all proteins in the MRM mode (10-100 ng/ml) as compared to the SIM mode (200-500 ng/ml), as a result of the improved selectivity.

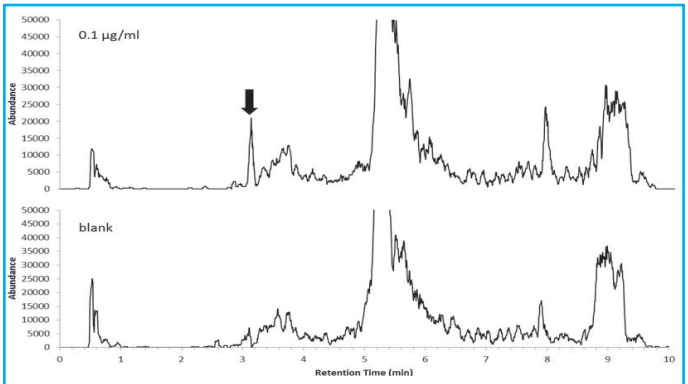

Figure 6: UPLC-MS chromatogram of Infliximab (upper) in mouse serum ultrafiltrate (Q-Exactive mass pectrometer). The Infliximab concentration was 0.1 µg/ml . The lower UPLC-MS chromatogram was obtained from blank mouse serum ultrafiltrate.

UPLC-MS analysis of intact apo-Transferrin in biological matrix (QqQ)

As stated, the goal of the performed experiments was the LC-MS analysis of intact proteins in a biological matrix rather than the development of an extraction procedure. Therefore, a "post-extraction" spiking approach was applied: calibration standards, prepared by spiking mouse serum ultrafiltrate at apo-Transferrin concentrations ranging from 0.05 µg/ml to 10 µg/ml, were analyzed using UPLC-MS/MS (MRM). The calibration results are presented in Figure 3. Quadratic regression analysis with weighting factor $1/x^2$ was performed. Without the use of an internal standard the accuracy was within the acceptance criterion of ≤15% (≤20% at lowest level) for at least 75% of the calibration standards. A mass chromatogram of apo-Transferrin in serum ultrafiltrate, spiked at a concentration level of 50 ng/ml, is presented in Figure 4 (upper chromatogram). Figure 4 (lower chromatogram) shows the blank serum ultrafiltrate. Apo-Transferrin had a retention time of 2.25 min and was just separated from an endogenous interference in the serum ultrafiltrate. It can be concluded that for the quantitative analysis of intact apo-Transferrin in a biological matrix, and most likely for all large proteins, very selective (immuno-affinity extraction) methods need to be developed.

UPLC-MS of intact proteins using HRMS

Full mass spectra of the proteins HSA, apo-Transferrin and Infliximab were acquired. The mass spectrum of Infliximab is presented in Figure 5. The most predominant ions were observed in the range from m/z 3713.79966 to m/z 4951.48913. Using a mobile phase of water/ACN (85/15, V/V) with TFA (0.1%), the mass spectrum of Infliximab was within the mass range of the Q-Exactive mass spectrometer (m/z up to 6000) but far beyond the range of the XEVO-TQS mass spectrometer (m/z up to 2000). Using the acquired mass spectra, UPLC-MS methods were set up.

The UPLC-MS analysis of Infliximab was investigated using full scan MS (m/z 3500-5000). In addition, the most intense and selective mass ranges were selected for each

protein to reduce the background signal. Summation of the selected m/z values improved the signal to noise ratio and, as a consequence, the LOD. The calibration standards, prepared in Tween 80/maltotriose (0.005%/0.5%) in water, were analyzed with the optimized conditions. The results are presented in Table 5. The calibration range in the table indicates the range in which the accuracy was within the acceptance criterion of ≤15% (≤20% at lowest level) for at least 75% of the calibration standards. Best results were obtained for the largest intact protein, Infliximab, because this protein could be measured at higher m/z values, thus eliminating interfering signals at lower m/z values. The LOD was approximately 0.05 µg/ml (Infliximab), 0.2 µg/ml (apo-Transferrin) and 0.5 µg/ml (HSA).

UPLC-MS analysis of intact proteins in biological matrix (HRMS)

The results of calibration standards, prepared in mouse serum ultrafiltrate, are presented in Table 5. For all three proteins the LOD was higher in serum ultrafiltrate because the processed matrix contained interfering endogenous compounds. In serum ultrafiltrate, the LOD was approximately 0.1 µg/ml (Infliximab), 0.5 µg/ml (apo-Transferrin) and 2 µg/ml (HSA). Calibration standards of Infliximab in serum ultrafiltrate were analyzed. Quadratic regression with weighting factor $1/x^2$ was applied. In the concentration range 0.1-5 µg/ml the accuracy was within the acceptance criterion of ≤15% (≤20% at lowest level) for at least 75% of the calibration standards without the use of an internal standard. A mass chromatogram of Infliximab in serum ultrafiltrate (100 ng/ml) is presented in Figure 6 (upper chromatogram). Figure 6 (lower chromatogram) shows a mass chromatogram of blank serum ultrafiltrate. By the optimization of the MS method an endogenous interference, which was observed close to the retention time of Infliximab, was eliminated. After the optimization no interference was observed at the retention time of Infliximab (3.11 min) in the selected mass range (Figure 6). However, for the quantitative analysis of intact Infliximab in a biological matrix a selective (immuno-affinity extraction) method has to be developed to eliminate interfering endogenous compounds, e.g. using immobilized TNF-α or even immobilized anti-idiotypic antibody, directed against Infliximab.

Conclusion and Discussion

This paper clearly demonstrates the quantitative possibilities of UPLC-MS analysis of intact proteins ranging from 12.4 kDa up to 148.5 kDa, using two state-of-the-art UPLC-MS systems. The triple quadrupole mass spectrometer (XEVO-TQS) enabled quantitative analysis of intact proteins at LOD levels ranging from approximately 10 to 100 ng/ml (MRM mode) whereas the LOD obtained with the high-resolution orbitrap mass spectrometer (Q-Exactive) was in the range 50-500 ng/ml. The orbitrap mass spectrometer offered the best sensitivity for the largest protein (Infliximab) whereas the triple quadrupole mass spectrometer gave the best LOD for the smallest protein (Cytochrome C).

On both mass spectrometers the calibration curves were non-linear, possibly due to adsorption of proteins in the low calibration range and ionization saturation/TRAP effects in the high calibration range. The calibration range was limited, i.e. approximately two orders of magnitude.

For quantitative purposes and in terms of variability the triple quadrupole mass spectrometer is recommended. If (additional) structural information is required, for example for the analysis of intact protein isoforms, the high resolution orbitrap mass spectrometer is the method of choice.

For the quantitative analysis of intact proteins in a complex biological matrix, such as plasma or serum, selective sample pre-treatment is required: for smaller intact proteins SPE can be applied but for the larger proteins immuno-affinity extraction methods need to be developed.

LBA, LC-MS (bottom-up approach) and LC-MS (top-down approach) are complementary techniques. Specific needs (such as selectivity, sensitivity, speed etc.) per application dictate the chosen technique. For example, the quantification of individual DAR species of an ADC in a biological matrix is best performed using LC-MS (top-down) [17]; LBA or LC-MS (bottom-up approach) do not offer the desired result. Furthermore, exploratory LC-MS based approaches (either top-down or bottom-up) may be more appropriate for the analysis of protein-based drugs in drug discovery (*in vitro* tests) and in early drug development (decision making). In later stages of drug development and clinical trials, however, fully validated, high-throughput and very sensitive LBA may be the best choice.

Protein	Molecular weight (kDa)	Matrix	Q-Exactive Calibration range (µg/ml)	Q-Exactive LOD (µg/ml)
HSA	66.5	Tween/maltotriose	0.50-10	0.50
		Serum ultrafiltrate	2.0-10	2.0
apo-Transferrin	79.5	Tween/maltotriose	0.20-10	0.20
		Serum ultrafiltrate	0.50-10	0.50
Infliximab	148.5	Tween/maltotriose	0.05-5.0	0.05
		Serum ultrafiltrate	0.10-5.0	0.10

Table 5: Calibration range and LOD of proteins analysed with UPLC and a high resolution mass spectrometer (Orbitrap, Q-Exactive). The calibration standards were prepared in Tween80 /maltotriose (0.005%/0.5%) in water or in mouse serum ultrafiltrate.

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