Feasibility Assessment of a Bioanalytical Method for Quantification of a 14.3 kDa Protein in Human Plasma using Tryptic Digestion LC-MS/MS without a Requirement for Antibodies

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A bioanalytical method for the quantification of a 14.3 kDa protein in human plasma has been developed using proteolytic digestion and LC-MS/MS quantification of a surrogate peptide. The technique provides an approach for the quantification of small protein therapeutics in biological fluids without the need for antibodies. Conventional LC-MS/MS sample handling or analysis procedures are not suitable for molecules of this size, and immunoassay methods may not yield adequate selectivity or sensitivity. Data is presented that demonstrates the selective quantification of a small protein at low nmol/L concentrations. The approach has future application in the bioanalysis of small protein therapeutics or small protein biomarkers particularly where immunoassay methods do not provide adequate selectivity, or where antibodies can not be raised.

1. Introduction

There has been a recent increase in the number of peptide therapeutics requiring quantitative LC-MS/MS bioanalytical assays. A number of papers have reviewed the issues encountered in developing bioanalytical assays for peptides [1-3]. The problems include adsorption, biological instability, difficult chromatography, low MS/MS response and protein or antibody binding. For peptides these challenges can often be overcome, however, for small proteins (>10000 Mwt) other approaches need to be considered. Small proteins demonstrate multiple charging under electrospray ionisation conditions with the formation of many multiply charged ions. Individual ions can be used for quantification, however, charge distribution means that the overall sensitivity is greatly reduced [4]. Additionally, the ratio of charge states can change depending on a number of factors, resulting in problems when monitoring only single ions [1]. Monitoring and summing the data for several ionisation states can increase response, but the endogenous background gains means that improvements to the signal to noise ratio are often negligible. Furthermore, larger molecules demonstrate a wide isotopic distribution which complicates the decision as to which ions to quantify, and limits the sensitivity further.

Proteins often display poor fragmentation efficiency under collision induced dissociation within the collision cell of a triple quadrapole mass spectrometer, due to the strength of the peptide bond and the position of the amino acid residues. At low collision energy very little fragmentation is observed whereas at higher collision energy the protein fragments completely to yield product ions of the small polypeptides, amino acids or their side chains. As all protein molecules entering the collision cell can also yield these same fragments, determining a selective but suitably abundant fragment for quantification can be challenging. Finally, proteins can demonstrate poor retention by reversed phase chromatography and poor solubility in organic solvents and therefore alternative methods are often required for sample handling, extraction and analysis.

The use of proteases to chemically digest a protein of interest into surrogate peptides which are then analysed by LC-MS/MS without the problems detailed above, has been documented in plasma [4, 5], serum [6], urine [7] and dried blood spots [8]. The problems associated with this approach are the limited levels of quantification restricted by poor proteolytic digestion efficiency and poor separation of the peptides of interest from those produced by digestion of endogenous plasma proteins. Often the limits of quantification of proteolytic digestion LC-MS/MS assays are inadequate

to support clinical or preclinical trials for high-potency protein therapeutics. The use of immunoaffinity enrichment or immunoprecipitation has been reported to achieve lower LC-MS/MS limits of quantification by concentrating the protein of interest and removing endogenous interferences [9-11], however, antibodies and immunoaffinity separations are both time consuming and costly. A method is presented for the quantification of Lysozyme, a 14.3 kDa protein, using a proteolytic digestion approach followed by LC-MS/MS. The method has a limit of quantification of 3.5 nmol/L and could be adapted for quantification of other small protein therapeutics or small protein biomarkers in biological fluids.

2. Materials and Methods

2.1 Chemicals and Reagents

All chemicals used in this work were obtained from Sigma Aldrich (Poole, UK), including Chicken egg white Lysozyme and trypsin (TPCK treated, non-acetylated). All solvents used were of HPLC grade or equivalent and all reagents were of 98% purity or greater. Solid phase extraction was performed using a positive pressure Varian CEREX (Palo Alto, CA, USA) and human plasma was purchased from BioChemed (Winchester, VA, USA).

2.2. LC-MS/MS methodology and optimisation

A pure standard of Lysozyme (5000 ng/mL) was digested by mixing 30 µL with denaturing solution 'A' (8 M urea $_{(aq)}$ containing 5 mM DTT, 120 µL) in a polypropylene tube and incubating at 60 °C for one hour. Iodoacetamide (200 mM, 20 µL) was added and the tubes were incubated in the dark for one hour. Digestion buffer (50 mM Tris pH 8 containing 1 mM $CaCl_2$, 1000 µL) and trypsin (500 µg/mL, 20 µL) were added and the tubes were incubated at 37 °C overnight to allow the digestion to proceed. Formic acid (20 µL) was added to stop the digestion. Peptides were purified using solid phase extraction (SPE) using a Waters Oasis HLB 10 mg plate (Milford, MA, USA). The sorbent was washed with water and peptides were eluted with 50/50/0.2 water/acetonitrile/formic acid (v/v/v) to produce an extract with suitable solvent composition for MS/MS infusion. Extracts were infused onto an Applied Biosystems API5000 instrument (Foster City, CA, USA) controlled by Analyst 1.4.2 software and surrogate peptides of Lysozyme were identified based on predictions from the protein sequence. A surrogate peptide of m/z 715.0 (z=2) was chosen using turbo ionspray, and fragmentation was assessed to identify the transition 715.0 – 804.7; MS/MS parameters were optimised for this transition (Table 1). A chromatographic method was developed using the Waters Acquity UPLC system (Milford, MA, USA) equipped with a Waters BEH-phenyl hexyl column 100 x 2.1 mm, 1.7 µm (Milford, MA, USA). Mobile phase A was water containing 0.01% trifluroacetic acid (TFA) and mobile phase B was acetonitrile containing 0.01% TFA. The flow rate was 0.5 mL/min and the column temperature set at 40 °C. A gradient of 10-40% mobile phase B was applied over 6.5 minutes. The conditions described gave a retention time for the selected surrogate peptide of approximately 2.0 minutes.

Mass Spectrometer Parameter	Setting
Ion Spray	5500 V
Temperature	700 °C
Curtain Gas	30
Desolvation Gas 1	50
Desolvation Gas 2	80
Collision Gas	4
Declustering Potential	100 V
Entrance Potential	10 V
Collision Energy	30 V
Cell Exit Potential	16 V

Table 1: Mass spectrometer settings.

2.3. Baseline plasma digestion approach (method one)

Human plasma (Li-Hep) was spiked with Lysozyme and samples were digested as described for pure standard solutions. The SPE method was further optimised for the surrogate peptide and so the sorbent was washed with 500 µL 95/5 water/acetonitrile (v/v) and surrogate peptides were eluted into a 96-well plate with 250 µL 75/25/2 water/acetonitrile/formic acid (v/v/v). The final extracts (20 µL) were injected onto the LC-MS/MS system.

2.4. Abundant protein depletion (method two)

Human plasma was spiked with Lysozyme and 500 µL of plasma was mixed with 2400 µL denaturing solution 'B' (6 M guanidine HCl $_{(aq)}$ plus 5 mM DTT) in a polypropylene tube. Each tube was incubated at 60 °C for one hour, then 50 µL iodoacetamide (500 µM) added and the tubes incubated for one hour in the dark. The samples were then loaded onto a Waters SPE plate (Oasis HLB 30 mg) equilibrated with methanol (1 mL) and water (1 mL). The sorbent was washed with 500 µL water and eluted with 500 µL water/acetonitrile/formic acid (80/20/1 v/v/v). Eluate (300 µL) was transferred to a clean polypropylene tube and 2500 µL digestion buffer added (96/4/0.2 50 mM Tris pH 8/6 M Guanidine HCl/500 mM $CaCl_2$ (v/v/v,)). To the tubes, 30 µL 1 mg/mL Trypsin was added and the tubes gently mixed during incubation at 37 °C overnight. Digested samples were subsequently processed by solid phase extraction and analysed as detailed for method one.

2.5. On-line solid phase extraction (method three)

Samples were processed as for method two. Eluant from the final SPE was diluted to a volume of 1 mL with 0.1% TFA in water containing 0.07% Triton X-100. The entire sample was loaded onto a Spark Holland Symbiosis system (Emmen, NL) using Hysphere C18 HD cartridges. MS/MS parameters were as described for method one. Loading, wash and elution steps on the Symbiosis were optimised for maximum cleanup and recovery of the surrogate peptide. The cartridge was equilibrated with 1 mL methanol and then 1 mL 0.1% TFA in water. The entire sample was then loaded. The cartridge was washed with 1 mL 95/5/0.1 water/acetonitrile/TFA (v/v/v) and elution achieved using the LC gradient. Mobile phase A was water containing 0.01% TFA and mobile phase B was acetonitrile containing 0.01% TFA. The column was a Waters X-Bridge phenyl-hexyl column (100 x 2.1 mm, 3.5 µm) with a flow rate of 0.5 mL/min and a column temperature of 40 °C. Mobile phase B (starting composition 10% v/v) was applied for 0.5 minutes, and a gradient of 10-30% mobile phase B applied over 5.5 minutes. The retention time of the selected surrogate peptide was approximately 3.0 minutes.

3. Results and Discussion

3.1. Method One

Method one yielded a limit of quantification of approximately 5000 ng/mL of Lysozyme in human plasma. The chromatogram (Figure 1) contains a peak for the surrogate peptide which is flanked by a large number of peaks derived from isobaric peptides of endogenous plasma proteins. A blank sample did not demonstrate significant peaks at the retention time of the surrogate peptide. Applying method one to process higher sample volumes (>30 µL) was unsuccessful as reduced digestion efficiency and lower recovery of the surrogate peptide was observed.

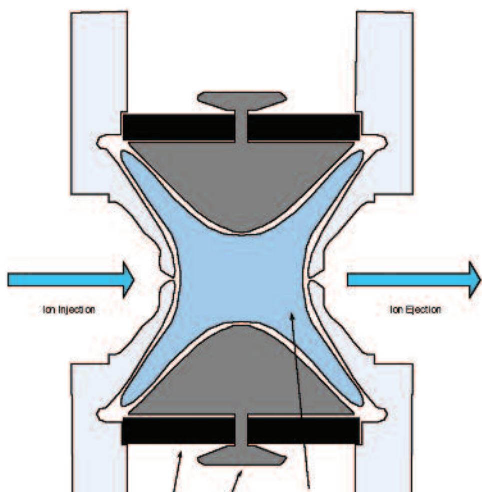

Figure 1: (A) Blank plasma chromatogram (B) Blank plasma spiked with 5000 ng/mL Lysozyme, extracted using method one. Arrows indicate the retention time of the surrogate peptide.

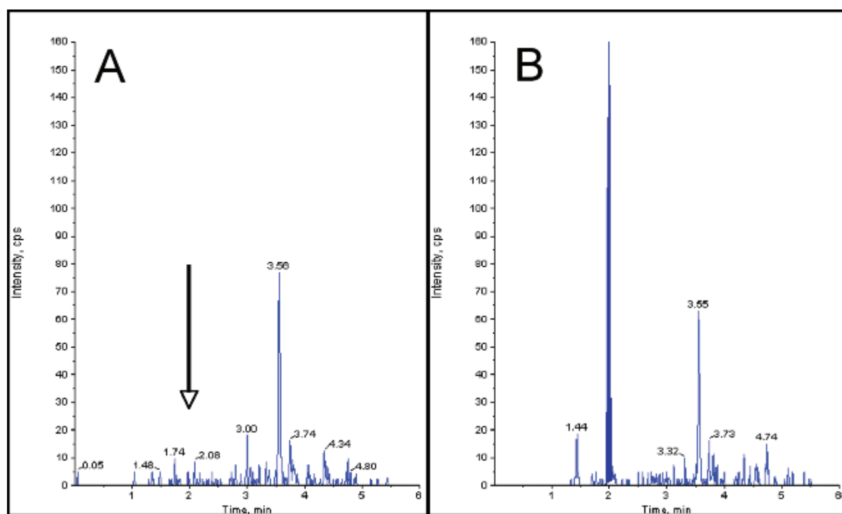

Figure 2: (A) Blank plasma chromatogram (B) Blank plasma spiked with 500 ng/mL Lysozyme, extracted using method two. Arrow indicates the retention time of the surrogate peptide in the blank sample.

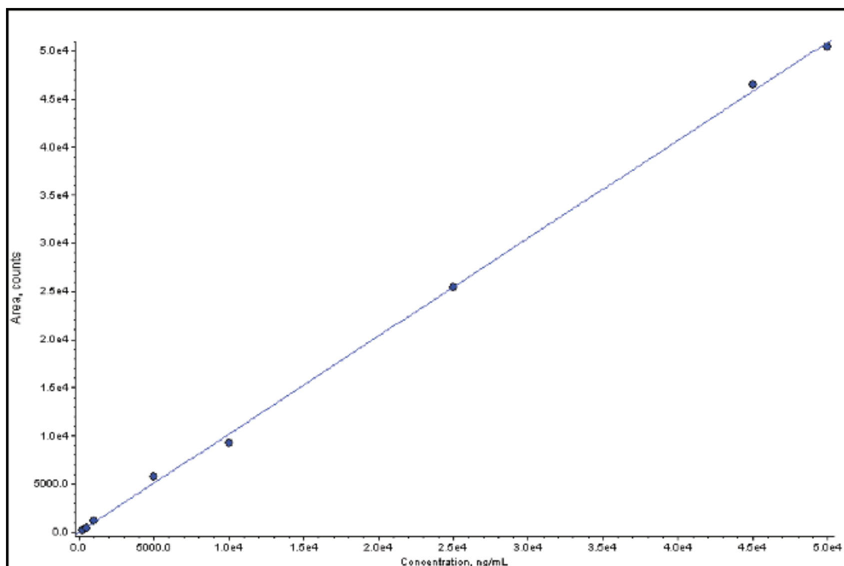

Figure 3: Calibration line (200-50000 ng/mL) obtained from method two (Linear 1/x). All calibration standards have an accuracy of 100% +/- 20%.

3.2. Method Two

To improve selectivity and process larger sample volumes it was necessary to deplete the large endogenous plasma proteins prior to digestion. Although several highly effective immunological approaches for achieving this are commercially available, they are only capable of processing low sample volumes (<20 µL). An approach was developed for high volume plasma depletion, taking advantage of the large mass (>50 kDa) of the most abundant plasma proteins; albumin and immunoglobulins. Solid phase extraction was used to deplete the plasma of the albumin and immunoglobulins by size exclusion prior to digestion. Protein depletion of plasma spiked with Lysozyme prior to denaturation yielded low recovery which appears to be due to protein binding of Lysozyme preventing its interaction with the SPE sorbent. Including a denaturation step prior to SPE allowed a plasma sample volume of 500 µL to be used and gave a lower limit of quantification of 500 ng/mL (Figure 2). A 16-fold increase in sample volume yielded a similar improvement to sensitivity over that obtained using method one. The non-specific peaks observed in Figure 1 are absent from Figure 2 confirming that they were tryptic peptides of large abundant plasma proteins. Using protein depletion overcame the loss in digestion efficiency observed at higher sample volumes. A calibration line (200-50000 ng/mL) was successfully constructed using method two (Figure 3).

3.3. Method three

To allow for greater loading of the final extract onto the LC-MS/MS system without compromising the chromatography or signal to noise ratio on-line solid phase extraction was performed. Extracts from method two were diluted to a volume of 1 mL with 0.1% $TFA_{(aq)}$ containing 0.07% Triton X-100. The aqueous dilution facilitated retention by reversed phase chromatography without breakthrough of the polar surrogate peptide; the TFA acts as an ion-pair reagent to enhance retention of the charged peptide and the Triton prevents adsorption of the peptide from the aqueous environment.

The entire sample was loaded onto the Symbiosis-MS/MS system, thus allowing approximately 12-fold greater loading compared to method two. A lower limit of quantification of 500 ng/mL (Figure 4), ten fold lower than method two, was achieved and indicated good recovery of the surrogate peptide from the on-line solid phase extraction cartridge. A calibration line (50-50000 ng/mL) was successfully constructed by this method (Figure 3).

4. Conclusions

The aim of this work was to develop a high throughput, cost effective and antibody-free proteolytic digestion-LC-MS/MS method for the quantification of a small protein at low nmol/L concentrations. The chosen test article was Chicken Egg White Lysozyme, a 14.3 kDa protein of comparable size to small protein therapeutics and biomarkers. Lysozyme is endogenous to human plasma and the human form is homologous to the chicken form, however,

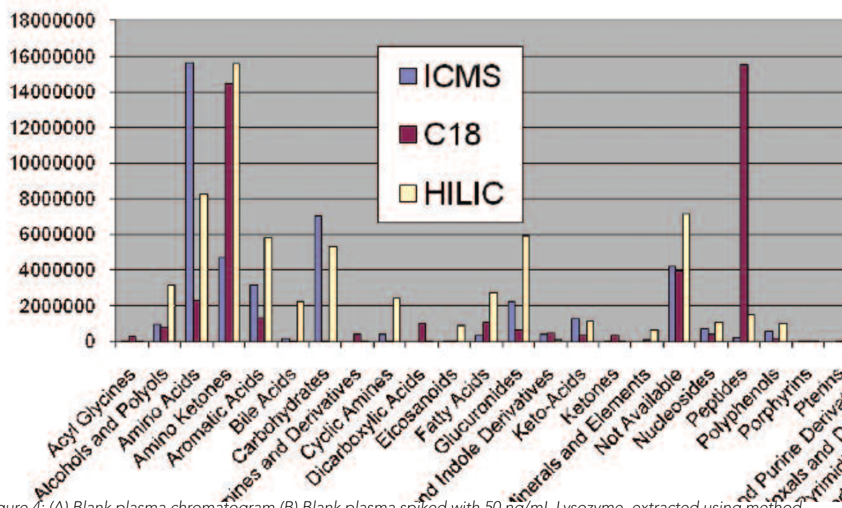

Figure 4: (A) Blank plasma chromatogram (B) Blank plasma spiked with 50 ng/mL Lysozyme, extracted using method three. Arrow indicates the retention time of the surrogate peptide in the blank sample.

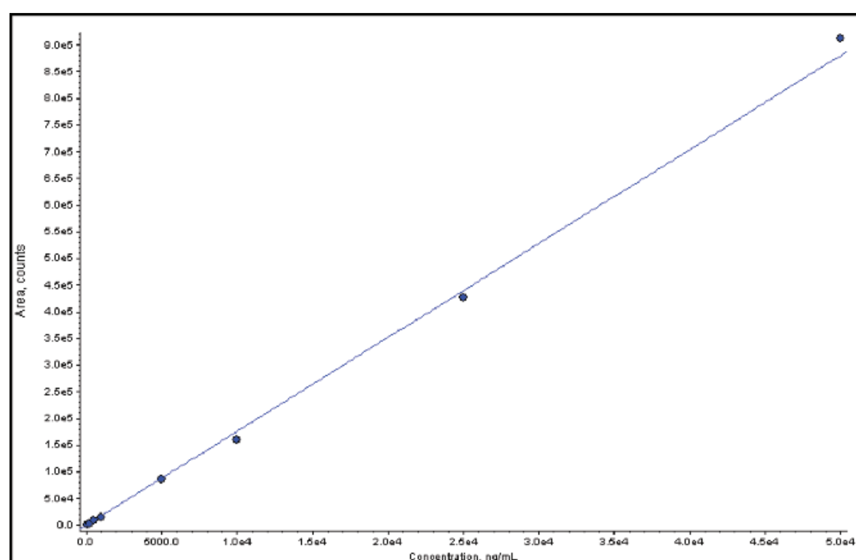

Figure 5: Calibration line (50-50000 ng/mL) obtained from method three (Linear 1/x). All calibration standards have an accuracy of 100% +/- 20%.

minor differences allow for the selectivity of the method to be demonstrated. The data presented demonstrates the feasibility of three approaches for the quantification of Lysozyme. A strategy to feasibly detect Lysozyme at low levels of quantification (50 ng/mL, 3.5 nmol/L) was achieved through implementation of abundant protein depletion and on-line solid phase extraction. The method development work for an assay such as this is quick and simple to perform and the method is high throughput, allowing for up to 96 samples per day to be processed. The work performed yielded good calibration lines in the absence of an internal standard. Proteolytic digestion-LC-MS/MS offers an approach for the bioanalytical quantification of small protein therapeutics or small protein biomarkers where antibodies are not available or where the selectivity of immunological approaches is not sufficient for the needs of the study.

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