Published in final edited form as:

Rapid Commun Mass Spectrom. 2009 March ; 23(5): 729–732. doi:10.1002/rcm.3926.

Accelerated tryptic digestion of proteins in plasma for absolute quantitation using a protein internal standard by liquid chromatography/tandem mass spectrometry

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Dear Editor

The emergence of protein-based therapeutics signals the paradigm shift in drug discovery and development in the last two decades. Today, the US Food and Drug Administration (FDA) has approved more than 130 proteins and peptides for clinical uses.¹ Absolute protein quantitation in complex biological samples has traditionally been conducted with immunoassay (IA).² Although IAs are both sensitive and high throughput, they are usually time-consuming and suffer from inaccurate results due to interferences in a complex matrix. In the proteomics arena, stable isotope labeling with amino acids in cell culture³ (SILAC) and absolute protein quantitation⁴ (AQUA) strategies have been widely used for protein quantitation. Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) is a mainstream technology in quantitative bioanalysis for small molecule drugs.⁵ The platform has the benefits of good specificity, reproducibility, high throughput, and wide dynamic range. For proteins of relatively low molecular weights, this strategy remains a viable option. Ji *et al.*⁶ used LC/MS/MS to quantify rk5, a small protein drug candidate of 10 464 Da, in monkey plasma to obtain its pharmacokinetic (PK) profile, where good linearity and low limit of quantitation were achieved. With the use of electrospray ionization (ESI), one disadvantage is that the intensity of a specific protein charge state is diluted or spread across all the charges. This is a rather profound issue especially for proteins of high molecular weights. Another promising strategy is to first digest a protein with an enzyme, commonly trypsin, followed by monitoring a signature peptide by LC/MS/MS. This was developed by Barr and colleagues in 1996 to quantitate proteins in pure solution.⁷ However, no internal standard (ISTD) was utilized to compensate for the variation that can occur during enzymatic digestion and subsequent sample preparation. The usage of an internal standard is a common practice in bioanalysis to achieve good assay accuracy and precision. Yang *et al.*⁸ reported the use of bovine fetuin as an internal standard in an assay development to quantitate a monoclonal antibody drug candidate using LC/MS/MS. Heudi and colleagues⁹ used an isotope-labeled protein version of the target as an internal standard and digested it simultaneously with the drug candidate. In both cases, good agreement was

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SUPPORTING INFORMATION

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seen from data obtained between LC/MS/MS and IAs. However, the two methods suffer greatly in terms of throughput, where the digestion took place overnight.

One potential solution is to speed up the enzymatic digestion in a high-throughput fashion. Previous reports^{10,11} showed that adding organic solvent to an enzymatic cleavage mixture can increase the reaction rate. Nevertheless, these experiments were carried out in controlled systems, where simple protein mixtures were dissolved in pure solvents. In the present study, we present an LC/MS/MS assay development for protein quantitation in complex matrix, i.e. human plasma, using accelerated trypsin digestion in the presence of methanol. The digestion was accomplished in 30 min in human plasma. Myoglobin (from horse skeletal muscle, Sigma-Aldrich) was used to characterize the accelerated trypsin digestion both in a buffer solution and in human plasma. An efficient LC method was developed using fused-core particles (C18) to separate signature peptides of interest from matrix components. Finally, somatropin (8.63 USP somatropin Units/vial, US Pharmacopeia), a human growth hormone, was used as the protein analyte to establish a linear calibration curve from 2.00 to 1000 µg/mL in human plasma, employing myoglobin as its internal standard.

The detailed sample preparation is as follows. For a typical accelerated digestion in plasma, 50 µL protein solutions made in 100 mM ammonium bicarbonate (pH ~7.8) were spiked into 50 µL human plasma treated with K3 EDTA in a 2-mL 96-well Axygen plate (Analytical Sales and Service, Pompton Plains, NJ, USA). Then 50 µL protein internal standard made in 100mM ammonium bicarbonate was added. Subsequently, 50 µL of trypsin (from bovine pancreas, TPCK-treated, 12500 units/mg protein, Sigma-Aldrich) made in 100 mM ammonium bicarbonate in various concentrations was added. The plate was sealed and vortexed briefly to ensure thorough mixing of trypsin and proteins. Methanol was then added to achieve various final concentrations and the plate was vortexed briefly. The plate was placed in a shaking water bath set at 37°C for 30 min or for a pre-determined time. The digestion was quenched by removing the plate to a wet ice bath and adding cold acetic acid to a final of concentration of 5% by volume. Overnight digestions (37°C) were carried out at the same protein concentration and trypsin-to-protein ratio as for accelerated digestion except that no methanol was present. For human plasma digestion, the plate was centrifuged at 3000 rpm at room temperature for 5 min to precipitate any undigested proteins and/or plasma proteins. The supernatant was analyzed directly. An Ascentis Express column (C18, 30 × 2.1 mm, 2.7 µm, Sigma-Aldrich) was used for peptide separations. The high-performance liquid chromatography (HPLC) system used was a Prominence system (Shimadzu Corp., Kyoto, Japan). A gradient was carried out between 0.2% formic acid in water (mobile phase A) and 0.2% formic acid in acetonitrile (mobile phase B). The gradient ran from 10% B to 35% B over 1.1 min, followed by a wash at 90% B for 1 min, and re-equilibration at 10% B for 1.3 min. An API 4000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with an ESI source was used throughout the study except for the high-resolution experiments. Accurate mass measurements were conducted on an LTQ Orbitrap (Thermo Scientific, San Jose, CA, USA) with a resolving power of 30 000. The scan time was 0.8 s. Xcalibur 1.0 (Thermo Scientific) was used to process high-resolution mass spectra obtained from the LTQ Orbitrap. Analyst v.1.4.2 software (Applied Biosystems/MDS SCIEX) was used to acquire and process all other data. A weighted ($1/x^2$) linear least-squares regression was used to generate the calibration curve.

Initial experiments were conducted to qualitatively compare the traditional overnight digestion and the accelerated digestion in the presence of methanol in buffer solution, i.e. 100 mM ammonium bicarbonate. The trypsin-to-substrate ratio was set to 1:50 (w/w). Myoglobin was used as a model protein. An API 4000, operated at unit resolution, and an LTQ Orbitrap, operated at a resolving power of 30 000, were used to identify the most

abundance species, m/z 636.5, observed commonly from both digestions. Accurate mass measurement from orbitrap and MS/MS from the LTQ (see Supplementary Figure 1, Supporting Information) confirmed that m/z 636.5 is a tryptic peptide of myoglobin, LFTGHPETLEK (amino acids 33–43). Accurate mass measurements from the LTQ Orbitrap were also used to confirm the identity of other myoglobin tryptic peptides. By comparing the accurate mass measurement obtained from the LTQ Orbitrap and the mass information from the possible myoglobin tryptic peptides from the *in silico* digestion, a total of ten peptides (Supplementary Table 1, see Supporting Information) were identified from both the accelerated digestion and the traditional overnight digestion. The ten peptides represent 72% sequence coverage of myoglobin. The results demonstrate that the accelerated digest protocol is qualitatively equivalent to an overnight digestion in terms of amino acid sequence coverage.

In quantitative LC/MS/MS assay development, LC separation plays a vital role in separating analyte(s) of interest from the complex matrix components. Due to the similarity between many protein therapeutics and endogenous interferences, it is critical to develop a selective LC method, where there is no interference appearing at the retention time of the analyte. This is the so-called selectivity in bioanalysis. The other important criterion in developing chromatography for bioanalysis is to accomplish the separation in a high-throughput manner. It is well known that fast separations for proteins and peptides on traditional bonded stationary phases are difficult to achieve mainly due to slow diffusion into and out of the stationary phases. Recently, fused-core silica particles, consisting of a solid core and a superficially porous shell, were introduced.^{12,13} These specially engineered semi-porous particles promise a smaller axial diffusion compared to completely porous particles and rapid mass transfer into and out of the stationary phase. Figures 1(a) and 1(b) show representative chromatograms of human plasma blank and myoglobin spiked in human plasma under accelerated digestion, respectively. The multiple reaction monitoring (MRM) transition monitored for the myoglobin peptide, LFTGHPETLEK, is 636.5→716.8. The results clearly demonstrate that the current LC method, utilizing fused-core particles, is selective in separating the myoglobin peptide from complex matrix components. Further, the separation was completed within 2 min with good peak shapes, supporting the advantage of fast mass transfer for peptide separations using fused-core particles.

There is a balance between the degree of substrate/protein denaturation, controlled by the percentage of methanol present in the digestion, and the biological activity of trypsin. Figure 2 shows the normalized intensities of the myoglobin tryptic peptide, LFTGHPETLEK, as a function of methanol content under accelerated digestion. Myoglobin concentration was kept the same, i.e. 200 $\mu\text{g/mL}$, at different methanol compositions. In human plasma, there are estimated to be over 20 000 different proteins and the plasma protein concentration is approximately 74 mg/mL .¹⁴ Compared to the total concentration of the plasma proteins, the concentration of myoglobin is insignificant in the spiked samples. Therefore, only the total plasma protein concentration is taken into consideration when the enzyme-to-substrate ratio is calculated. The trypsin-to-substrate ratio was arbitrarily set at 1:50 (w/w). Since the same volumes of human plasma and trypsin were added, the trypsin concentration was made at 1.48 mg/mL in 100 mM ammonium bicarbonate. Three replicate experiments were carried out at each methanol concentration. The optimal concentration of methanol in the digestion solution was determined by varying the methanol proportion from 20 to 80% with a 15% increment. As shown in Fig. 2, 50% methanol generated the highest response for the myoglobin tryptic peptide, LFTGHPETLEK. The response is minimal and nearly stagnant when the methanol concentration is below 35%, which is likely due to insufficient denaturation of the substrate protein. In comparison, the response of LFTGHPE-TLEK was enhanced by nearly 5-fold when the methanol concentration was increased to 50% from 35%. The results suggest that higher concentrations of methanol lead to a further unfolding

of the protein, thus further exposing its reaction sites to trypsin. At even higher organic concentration (>65%), the response of the peptide decreases, probably ascribed to the loss of trypsin biological activity. It is also possible that myoglobin is co-precipitated along with more abundant plasma proteins. The plasma protein concentration was diluted 4 times prior to the addition of methanol, which certainly helps to minimize co-precipitation of target proteins with other major plasma proteins. Thus, 50% methanol is determined to be the optimal condition for the accelerated tryptic digestion in plasma.

Traditional overnight digestions typically use a relatively small amount of trypsin, i.e. 1:100 enzyme/substrate ratio (w/w). To investigate the effect of enzyme/substrate ratio on the yield of tryptic peptides, both myoglobin and somatropin were digested under accelerated conditions. Both proteins were made at a concentration of 200 µg/mL and were spiked into human plasma prior to the digestion. A total plasma protein concentration of 74 mg/mL was used. Trypsin was made at different concentrations in 100 mM ammonium bicarbonate, i.e. 0.1, 0.5, 1, 5, and 10 mg/mL. This corresponds to enzyme/substrate ratios of 740:1, 148:1, 74:1, 14.8:1, and 7.4:1 (w/w) in the final digestion, respectively. All experiments were conducted with a final methanol concentration of 50% (v/v). Shown in Fig. 3 are the normalized abundances for peptides LFTGHPETLEK and LFDNAMLRL, corresponding to myoglobin and somatropin, respectively, as a function of trypsin concentrations. The number at each trypsin concentration level represents the ratio of substrate/enzyme (w/w) in the final digestion. For example, at the lowest trypsin concentration, 0.1 mg/mL, the ratio of protein to trypsin is roughly 740:1. The sensitivities of both peptides increase modestly with the trypsin concentration in the range of 0.1 to 5 mg/mL. In comparison, there is approximately a 6-fold and 3-fold sensitivity enhancement for myoglobin and somatropin peptides when the trypsin concentration was doubled from 5 to 10 mg/mL. Since regular reagent-grade trypsin was used,⁶ the cost is actually very affordable for accelerated digestion for plasma samples. Generally, the higher the trypsin concentrations, the higher the yield of tryptic peptides under the accelerated digestion regime. However, the gain in throughput significantly outweighs the extra trypsin used for the accelerated digestion. In terms of throughput, there is at least 24-fold gain using the current rapid digestion protocol (30 min) assuming that overnight digestion takes 12 h. Obviously, the sample preparation for protein quantitation in plasma will no longer be a rate-limiting step in the bioanalytical method development and validations. The ultimate benefit is fast turn-around time for protein sample analysis to support drug development programs.

To test the validity of the accelerated digestion protocol for quantitative bioanalysis, a calibration curve was established using somatropin as the analyte and myoglobin as its analogous protein internal standard to account for the variability incurred during sample preparation. The solid circles in Fig. 4 were obtained experimentally from seven calibration standards, whereas the line is the linear regression of the experimental data. The calibration curve is linear over the range of 2.00–1000 µg/mL for somatropin with an R^2 value of 0.9985. Good linearity suggests that the strategy of employing an analogous protein as the internal standard works. Meanwhile, excellent linearity over almost three orders of magnitude strongly supports the reliability of the current accelerated enzymatic digestion for LC/MS/MS quantitation of proteins in plasma.

In all, a method for accelerated trypsin digestion (30 min) in a complex matrix, i.e. human plasma, was developed for protein quantitation using LC/MS/MS. Increased enzymatic digestion was achieved with the assistance of methanol, and 50% methanol in the final plasma samples was found to be optimal. The accelerated digestion generally requires more trypsin to achieve higher peptide yields compared to the conventional overnight digestion. Nevertheless, reagent-grade trypsin is sufficient to perform the digestion in human plasma. A calibration curve with good linearity ($R^2 = 0.9985$) was established for somatropin from 2

to 1000 µg/mL using myoglobin as its analogous internal standard under the accelerated protein digestion protocol. This rapid digestion protocol opens new doors for reliable quantitation of protein therapeutics in plasma by LC/MS/MS in a high-throughput manner. This method may also be applicable to quantitation of other proteins, such as disease biomarkers and other proteins of interest.

Supplementary Material

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Acknowledgments

The authors would like to thank Mary Pelzer and Hongliang Jiang for thoughtful discussions and Austin C. Li for the assistance in setting up the LTQ Orbitrap. A University of Wisconsin Distinguished Graduate Fellowship through the Pharmacy School in the name of Dr. Kenneth Connors supported C. M. Schmerberg in part while conducting this work.

References

1. Leader B, Baca QJ, Golan DE. Nat Rev Drug Discov 2008;7:21. [PubMed: 18097458]
2. DeSilva B, Smith W, Weiner R, Kelley M, Smolec J, Lee B, Khan M, Tacey R, Hill H, Celniker A. Pharm Res 2003;20:1885. [PubMed: 14661937]
3. Ong SE, Mann M. Methods Mol Biol 2007;359:37. [PubMed: 17484109]
4. Kirkpatrick DS, Gerber SA, Gygi SP. Methods 2005;35:265. [PubMed: 15722223]
5. Ackermann BL, Berna MJ, Eckstein JA, Ott LW, Chaudhary AK. Ann Rev Anal Chem 2008;1:357.
6. Ji QC, Rodila R, Gage EM, El-Shourbagy TA. Anal Chem 2003;75:7008. [PubMed: 14670064]
7. Barr JR, Maggio VL, Patterson DG Jr, Cooper GR, Henderson LO, Turner WE, Smith SJ, Hannon WH, Needham LL, Sampson EJ. Clin Chem 1996;42:1676. [PubMed: 8855153]
8. Yang Z, Hayes M, Fang X, Daley MP, Ettenberg S, Tse FL. Anal Chem 2007;79:9294. [PubMed: 17999465]
9. Heudi O, Barteau S, Zimmer D, Schmidt T, Bill K, Lehmann N, Bauer C, Kretz O. Anal Chem 2008;80:4200. [PubMed: 18465883]
10. Arsene CG, Ohlendorf R, Burkitt W, Pritchard C, Henrion A, O'Connor G, Bunk DM, Güttler B. Anal Chem 2008;80:4154. [PubMed: 18447320]
11. Russell WK, Park ZY, Russell DH. Anal Chem 2001;73:2682. [PubMed: 11403317]
12. Gritti F, Guiochon G. J Chromatogr A 2007;1166:30. [PubMed: 17719592]
13. Destefano JJ, Langlois TJ, Kirkland JJ. J Chromatogr Sci 2008;46:254. [PubMed: 18334092]
14. Van Slyke DD, Hiller A, Phillips RA, Hamilton PB, Dole VP, Archibald RM, Eder HA. J Biol Chem 1950;183:331.

Figure 1. Representative chromatograms for a myoglobin tryptic peptide, LFTGHPETLEK, obtained from (a) human plasma blank and (b) myoglobin spiked in human plasma.

Figure 2.
Effect of methanol concentration on the digestion of myoglobin in human plasma (n = 3).

Figure 3.

Effect of trypsin concentration (i.e. enzyme-to-substrate ratio, see text for details) on the digestion efficiency of somatropin and myoglobin ($n = 3$). Each peptide is normalized to its highest intensity (100%) in five trypsin concentrations.

Figure 4. A fresh-spiked somatropin calibration curve (2.00–1000 $\mu\text{g/mL}$) in human plasma using myoglobin as the internal standard using accelerated digestion.