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Quantification of human serum transferrin using liquid chromatography–tandem mass spectrometry based targeted proteomics

Ying Yu [a], Jinhui Xu [a], Yuan Liu [b], Yun Chen [a,*]

[a] Nanjing Medical University, Nanjing 210029, China
[b] First Affiliated Hospital of Nanjing Medical University, Nanjing 210006, China

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ABSTRACT

Currently, the absolute quantification of human transferrin (hTRF) is based on several techniques other than mass spectrometry. Although these techniques provide valuable information on protein levels and can be extremely sensitive, they often lack the specificity and reproducibility that can be provided by mass spectrometry. In this study, a liquid chromatography–tandem mass spectrometry (LC/MS/MS) based targeted proteomics assay was developed and validated for the determination of transferrin in human serum. We selected the tryptic peptide 108EDPQTFYYAVAVVK121 as the surrogate analyte for quantification and used a stable isotope-labeled synthetic peptide with this sequence as an internal standard. Sample cleanup and enrichment were achieved using solid phase extraction. The validated calibration range was from 500 to 5000 ng/mL. The intra- and inter-day precisions were less than 4.9% and 9.0%, respectively. The bias for the quality control (QC) samples was less than 5.4%. Finally, this assay was successfully applied to the quantitative analysis of transferrin in clinical samples. The obtained values were assessed by independently measuring transferrin in the same samples using a commercially available immunoturbidimetric assay. As a result, the absolute concentrations determined by the LC/MS/MS assay compared well with those obtained with the immunoturbidimetric method; however, the LC/MS/MS assay afforded more reliable transferrin values at low concentrations.

1. Introduction

Human transferrin (hTRF) is the principal iron-transporting protein in the body. Its main function is to bind circulating iron and transport it to a range of cell types [1]. Thus, the absolute quantification of hTRF is usually performed in clinical situations where ion metabolism is evaluated (e.g., anemia [2]). In addition, a number of studies have indicated that hTRF in serum and other body fluids is a potential biomarker for the early detection of certain cancers, such as colon cancer [3] and oral cancer [4]. These findings provided the incentive for developing an accurate and precise cancer-screening assay.

To date, several assays have been developed and validated for the measurement of hTRF. Most utilize techniques other than mass spectrometry, such as western blot analysis, enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays (RIAs). While these techniques provide valuable information on protein levels and can be extremely sensitive, they often lack the specificity and reproducibility that mass spectrometry can offer [5]. Additionally, poor agreements between different assays for protein quantification have been reported [6,7]. Because of these issues, mass spectrometry has been developed and applied as an alternative technique.

Mass spectrometry techniques, such as the "shotgun" strategy, have been used as powerful tools for the relative quantitative measurement of proteins on a proteomic scale [8,9]. However, these types of approaches are generally non-targeted, i.e., in each measurement, they stochastically sample a fraction of the proteome that is usually biased toward the higher end of the abundance scale [10,11]. Therefore, there is a growing interest in targeted proteomics where a limited number of proteins are pre-selected and quantified [12,13].

The underlying principle of targeted analysis is to specifically detect and determine a protein of interest at the peptide level. The peptides are generated by proteolytic digestion of the targeted protein and serve as surrogate analytes. Selected reaction monitoring (SRM or MRM) on a triple quadrupole instrument is generally employed for quantification, and liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) assays are developed to detect fragment ion signals from those unique peptides [13].

* Corresponding author at: Department of Pharmacology, Nanjing Medical University, 140 Hanzhong Road, Nanjing 210029, China. Tel.: +86 25 86862764; fax: +86 25 86863165.
E-mail addresses: ychen@njmu.edu.cn, yun.chen.chem@gmail.com (Y. Chen).

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Then, the absolute quantification of proteins in biological samples can be accomplished. Recently, there has been increasing support for targeted proteomics with high sensitivity, selectivity and wide dynamic range [14].

In this report, we demonstrate that proteolysis, followed by the monitoring of a specific enzymatic cleavage fragment using LC/MS/MS, can be used to absolutely quantify serum hTRF. A stable isotope-labeled synthetic peptide was used as an internal standard, and the assay was validated. Finally, this assay was applied to the quantitative analysis of hTRF in clinical samples. The resulting values were compared to those obtained with a validated immunoturbidimetric method.

2. Materials and methods

2.1. Chemicals and reagents

The hTRF and human serum albumin (HSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium bicarbonate (NH_4HCO_3) was obtained from Qiangshun Chemical Reagent Co. Ltd. (Shanghai, China). DL-dithiothreitol (DTT) and iodoacetamide (IAA) were both supplied by Sigma–Aldrich (St. Louis, MO, USA). Sequencing-modified grade trypsin was purchased from Promega (Madison, WI, USA). Phosphate buffered saline (PBS) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Acetonitrile (ACN) and methanol were obtained from Tedia Company, Inc. (Fairfield, OH, USA). Trifluoroacetic acid (TFA) and formic acid (FA) were provided by Aladdin Chemistry Co. Ltd. (Shanghai, China) and Xilong Chemical Industrial Factory Co. Ltd. (Shantou, China), respectively. Water was purified and deionized with a Milli-Q system manufactured by Millipore (Bedford, MA, USA).

2.2. Preparation of stock solutions, calibration standards and quality controls (QCs)

The hTRF was accurately weighed, and a 1 mg/mL stock solution was prepared by dissolving the protein in deionized water. The solution was stored at −20 °C in a brown glass tube to protect it from light. In this report, an isotope-labeled synthetic peptide was used as an internal standard. Details about the selection of the internal standard are described below. The synthetic peptide was also weighed, and a 5 μg/mL stock solution was prepared in deionized water. A 1000 ng/mL internal standard solution was prepared by diluting the stock solution with a ACN:water mixture (50:50, v/v) containing 0.1% FA.

The hTRF calibration standards were prepared by a serial dilution of the stock solution using 5% HSA in phosphate buffered saline (PBS) as the matrix. The concentrations of the calibration standards were 500, 1000, 1750, 2500, 3000, 3750 and 5000 ng/mL. The QC standards (i.e., lower limit of quantification (LLOQ), low QC, mid QC and high QC) were prepared at 500, 1500, 2500 and 4000 ng/mL, respectively, in the same matrix and frozen prior to use.

2.3. Serum depletion and in-solution tryptic digestion

Albumin and IgG were depleted from the samples using the ProteoPrep® Blue Albumin and IgG Depletion Kit ("ProteoPrep Blue," Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Then, 100 μL of each depleted sample was mixed with 50 μL of 50 mM NH_4HCO_3. Denaturation was performed at 95 °C for 8 min. Subsequently, the protein was reduced with an addition of 50 mM DTT to a final concentration of 10 mM and incubated at 60 °C for 20 min. The sample was then alkylated by adding 400 mM IAA to a final concentration of 50 mM and incubated at room temperature for 6 h in the dark. Finally, 35 μg of sequencing grade trypsin was added, and the sample was incubated at 37 °C for 24 h. The reaction was stopped by adding 10 μL of 0.1% TFA. Then, 100 μL of the internal standard solution was added to the tryptic peptide mixture before transferring it into an Oasis HLB cartridge (60 mg/3 mL Waters, Milford, MA, USA) that was preconditioned with 3 mL ACN and 3 mL deionized water. After the sample was loaded, the cartridge was washed with 2 mL of water and 2 mL of ACN:water (50:50, v/v) and eluted with 1 mL of 100% ACN. Finally, the eluent was evaporated to dryness and then resuspended in 100 μL of ACN:water (50:50, v/v) containing 0.1% FA.

2.4. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

A Bruker MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany) was used to identify and acquire the relative abundance of the tryptic peptides generated from the protein digestion. The analysis was performed at 372 nm. Positive ion MALDI mass spectra were obtained using a radio frequency of 200 Hz, a MS/MS acceleration voltage of 21 kV and a m/z range of 500–4000. A 4 μg/μL α-cyano-4-hydroxycinnamic acid (α-HCCA) matrix solution was prepared in ACN:water (70:30, v/v) containing 0.1% TFA. A 1 μL sample was spotted onto a target plate and allowed to dry at room temperature. Then, 0.05 μL of the matrix solution was applied to the same position and dried before detection. Using MASCOT, a human protein subset from the Swiss-Prot protein database was used to search for the resulting mass data.

2.5. Stable isotope-labeled peptide internal standard

The most intense hTRF tryptic peptides identified in the MALDI-TOF mass spectra were selected to verify its specificity for hTRF. Their sequences were searched for exact matches using BLASTP against the genome-derived human Ensembl peptides in Ensembl Blastview (www.ensembl.org/Homo_sapiens/blastview) [9]. The sequence unique to hTRF was used to design the stable isotope-labeled internal standard. The stable isotope-labeled amino acid was supplied by Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) and the isotope-labeled peptide was developed by ChinaPeptides Co., Ltd. (Shanghai, China).

2.6. LC/MS/MS

An Agilent Series 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) and a 6410 Triple Quad LC/MS mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) were used.

The liquid chromatography separations were performed on a hypersil gold column (3 μm, 20 mm × 2.1 mm; Thermo Fisher Scientific, USA) at room temperature. The mobile phase consisted of solvent A (0.1% FA in water) and solvent B (0.1% FA in methanol). A linear gradient with a flow rate of 0.3 mL/min was applied in the following manner: B 10% (0 min) → 10% (1 min) → 90% (4 min) → 90% (8 min) → 10% (9 min). The injection volume was 10 μL.

The mass spectrometer was interfaced with an electrospray ion source and operated in the positive MRM mode. Q1 and Q3 were both set at unit resolution. The flow of the drying gas was 10 L/min, while the drying gas temperature was held at 350 °C. The electrospray capillary voltage was optimized to 4000 V. The nebulizer pressure was set to 45 psi. The data were collected and processed using the Agilent MassHunter Workstation Software (version B.01.04).

2.7. Method validation

Method validation involves linear range, accuracy, precision, limit of quantification (LOQ) and recovery. The detailed procedures

and the acceptance criteria used to validate the assay have been described in a number of publications [15].

2.8. Comparative study

To examine the applicability of the developed assay, twenty human serum samples (separated from ~5 mL blood) were obtained from volunteers and were stored at −20 °C. This study was approved by the institutional review board of Nanjing Medical University. Twenty volunteers were consecutively recruited between January 2011 and December 2011 at the First Affiliated Hospital of Nanjing Medical University, Nanjing, China (ten men and ten women with a mean age and range of 32.5 and 15–45 years, respectively). They were biologically unrelated, but they all belonged to the Han Chinese ethnic group from the Jiangsu province in China. In addition, ten of these volunteers, to their knowledge, were healthy and had no reason to consult their local doctors during the preceding 12 months. The others were patients who were newly diagnosed with diabetes, breast cancer, nephropathy or cirrhosis. Informed consent was obtained from the subjects, and the collected samples were prepared and analyzed using the procedure described above. Finally, the results were compared with those of a commercially available immunoturbidimetric assay. The immunoturbidimetric assay was performed at the First Affiliated Hospital of Nanjing Medical University on a BN II nephelometer (Dade Behring, Marburg, Germany) with the corresponding Dade Behring reagents for serum hTRF.

3. Results and discussion

3.1. Digestion efficiency

To generate a high-quality SRM assay, an efficient and specific trypsin digestion of proteins is first required [16]. Digestion strategies may employ organic solvents, heat, chaotropes or surfactants to denature the proteins before digestion to render more of the protein's structure accessible to the proteolytic enzyme [17]. Organic solvents and heat were evaluated in this study because chaotropes and surfactants (e.g., urea, SDS and guanidine HCl) can inactivate proteases at high concentrations required for denaturation and compete with peptide ions for adsorption on stationary phases during liquid chromatography and for charge during mass spectrometry. The results of the HPLC data indicated that approximately 99.6% and 99.9% of hTRF was digested after denaturation with heat or ACN (Figure 1S), respectively. Because the removal of ACN might be required prior to reduction and alkylation of the disulfide bonds, heat was selected to denature hTRF.

Notably, a number of other factors can also affect the digestion efficiency, such as digestion temperature, reaction time, enzyme to substrate ratio and digestion buffer. The decision to optimize these factors is determined by whether complete digestion is required for the study. In traditional proteomics, protein identification benefits greatly from ensuring complete proteolytic digestion across the proteome [18]; however, the targeted proteomics approach may only require complete cleavage of the surrogate peptides. Therefore, these factors were not evaluated in this study.

It should be noted that the HPLC results reported above do not indicate the full recovery of the surrogate peptides. It is not easy to evaluate the efficiency of the proteotypic peptide released from the proteolytic digestion. Several studies have employed synthesized peptides, containing the same sequence of surrogate peptides, to mimic a piece of the targeted protein [19,20]. However, from our point of view, this process does not take the steric hindrance of enzymatic digestion into account. The catalytic site may be buried within a large protein structure. Therefore, we applied another strategy to estimate the digestion efficiency. Using 108EDPQTFYYAVAVVK121 as a surrogate analyte, peptide mapping of hTRF yielded its corresponding molecular ion at *m/z* 1629.8 and larger peptide fragments with 1–6 missed cleavages (i.e., *m/z* 5564.8, *m/z* 1757.9, *m/z* 5692.9, *m/z* 6486.3, *m/z* 2934.4, *m/z* 6614.3, *m/z* 6614.3, *m/z* 6869.4, *m/z* 3119.6, *m/z* 6742.4, *m/z* 7790.9, *m/z* 7054.6, *m/z* 7919.0, *m/z* 7976.0 and *m/z* 8104.1, Table 1S). Their multiple charged ions were also monitored. By assuming that the mass response of each peptide fragment was the same, the peptide-bond cleavage for the generation of 108EDPQTFYYAVAVVK121 was 96% complete. Notably, most of the missed cleavage fragments were unobservable in the MALDI mass spectrum, but they were screened and validated by LC/MS/MS with selected ion monitoring (SIM). In addition, the efficiency of the reduction and alkylation were also evaluated and estimated to be approximately 95% and 77%, respectively.

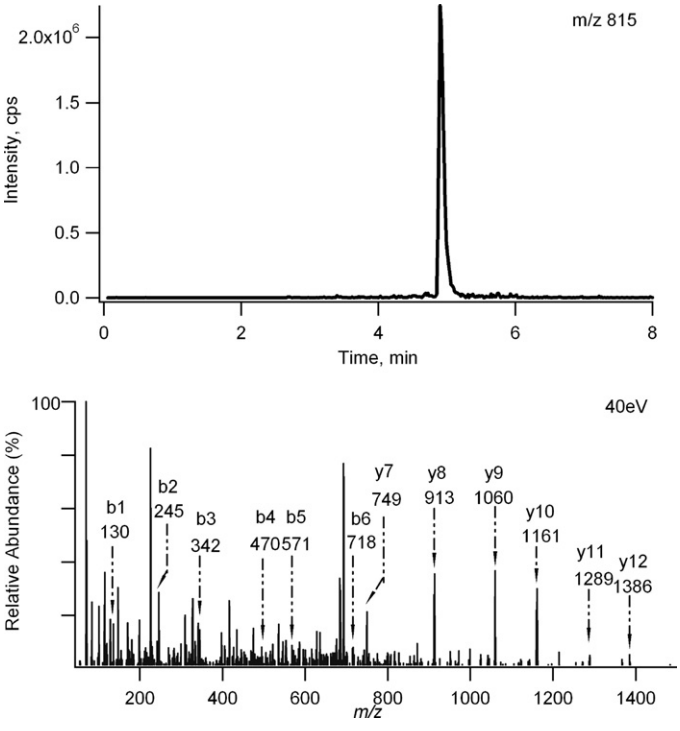

Fig. 1. The extracted ion chromatogram and the product ion spectrum of 108EDPQTFYYAVAVVK121.

3.2. Electrospray response of tryptic peptides

Another prerequisite for developing a LC/MS/MS-based targeted proteomics assay is the selection of tryptic peptides that could provide specificity and an adequate response. Because ionization suppression may be caused by other co-eluting peptides, it is difficult to predict the electrospray ionization efficiency of a peptide in a complex mixture. Therefore, a full scan LC/MS/MS analysis was performed to identify the tryptic peptide in the greatest abundance. The most intense peptide was the doubly charged ion of 108EDPQTFYYAVAVVK121. Its presence was also confirmed in the MALDI mass spectrum. In addition, this sequence was found to be unique to hTRF (accession no. P02787 (TRFE_HUMAN), gi: 136191) via a BLAST search, suggesting that it could be used to specifically quantify serum hTRF. However, it should be noted that this peptide sequence is also observed in other primates. Finally, this selected peptide was also consistent with the previous evidence (http://www.peptideatlas.org) [21].

The extracted ion chromatogram and product ion spectrum of 108EDPQTFYYAVAVVK121 are shown in Fig. 1. The characteristic

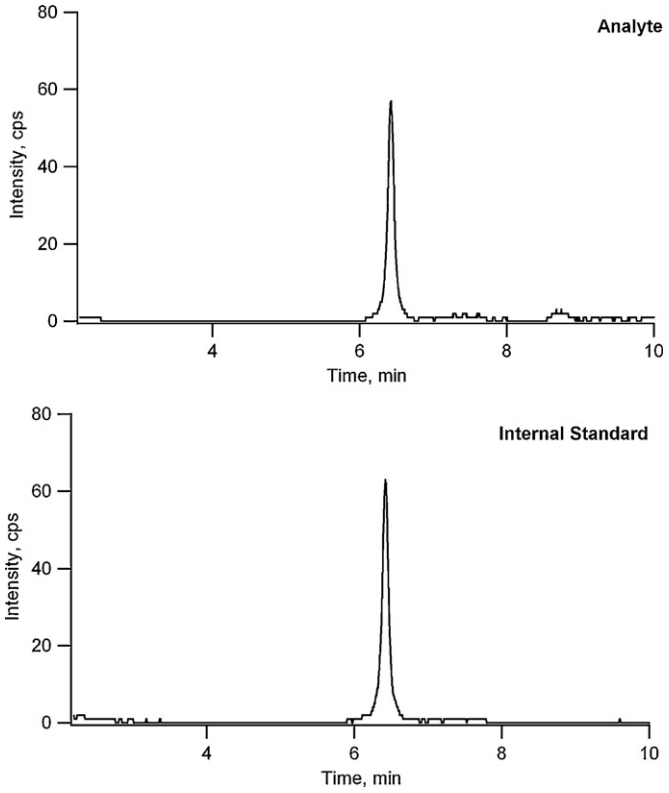

Fig. 2. The LC/MS/MS chromatograms of 108EDPQTFYYAVAVVK121 and the stable isotope-labeled internal standard.

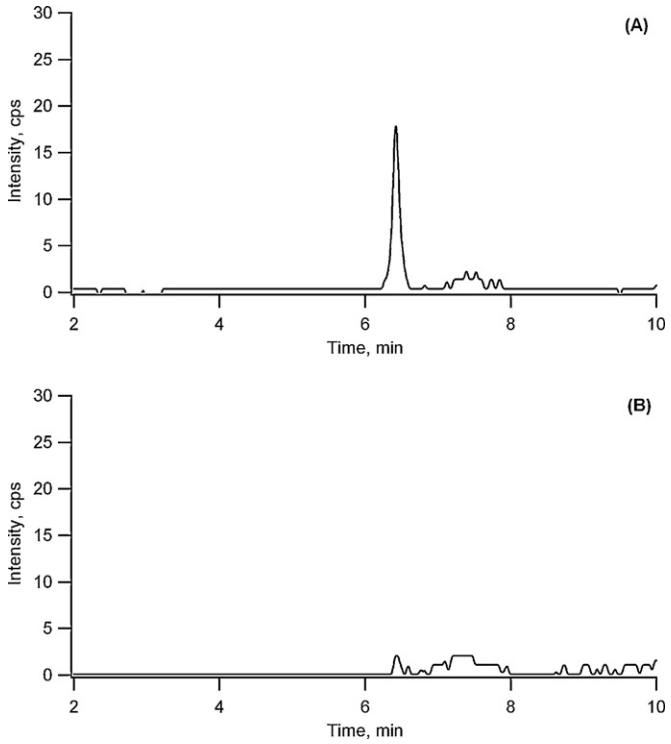

Fig. 3. The LC/MS/MS chromatograms of LLOQ (A) and blank matrix (B). The internal standard is not shown to simplify the data presentation.

sequence-specific b ions (m/z 130(b1), m/z 245(b2), m/z 342(b3), m/z 470(b4), m/z 571(b5) and m/z 718(b6)) and y ions (m/z 749(y7), m/z 913(y8), m/z 1060(y9), m/z 1161(y10), m/z 1289(y11) and m/z 1386(y12)) were indicative of this peptide. Therefore, a synthetic stable isotope-labeled peptide, EDPQTFYYAVAV*V*K, was prepared. A stable isotope-labeled valine containing an added mass of 8 Da from deuterium was coupled to the peptide sequence at positions 12 and 13 to yield a total molecular mass shift of 16 Da from the non-labeled peptide and a monoisotopic molecular mass of 1645 Da. In addition, its product ion spectrum was acquired and validated (data not shown). The retention times of 108EDPQTFYYAVAVVK121 and its isotope-labeled peptide were identical (~6.4 min), implying that the deuterium isotopic effect on the retention time was negligible (Fig. 2).

We also investigated the location of several of the most abundant peptides (EDPQTFYYAVAVVK, DGAGDVAFVK, DSGFQMNQLR, etc.) in the 3-dimensional structure of hTRF. The 2HAU apo-human serum transferrin structure was downloaded from the RCSB Protein Data Bank [22]. The PDB protein workshop program was used to visualize and highlight the location of these peptides. Interestingly, these intense peptides (Figure 2S) are all located on the external part of the molecule, as previously reported [19,23]. This finding could suggest that trypsin tends to attack exposed polypeptides on the exterior of the protein. Nevertheless, this illustration of the peptides' locations may help with selecting a peptide for protein quantification.

3.3. LC/MS/MS assay development and validation

As previously reported, an important factor needed to achieve good quantification of proteins is to generate high-quality MRM [16]. The MRM assays are generally developed on a triple quadrupole instrument to detect fragment ion signals arising from unique surrogate peptides. The y-series fragment ions are usually employed to establish the MRM transitions for quantification [24]. However, the transition that had the best signal-to-noise and LOQ for hTRF in this study was afforded by the immonium product ion of the proline residue at position 3. This characteristic mass pattern was also observed in the stable isotope-labeled internal standard. The enhanced intensity of the immonium product ion could be attributable to the terminal effect [25] and its neighboring aspartic acid residue [26]. In fact, other sensitive peptide analyses have been previously reported in our laboratory. For example, we demonstrated that structurally distinctive immonium ions are formed in high abundance [27–29].

Using the transitions of m/z 815 → 70 and m/z 823 → 70 (internal standard), an hTRF LC/MS/MS assay was developed and validated. Solid phase extraction was selected as the technique of choice for sample cleanup and enrichment in this study because it has shown great promise for sample cleanup. As shown in Figure 3S, a good peak shape was achieved after the extraction. The calibration curve was constructed using a weighted linear regression model with a weighting factor of $1/x^2$. The relative peak area ratio of the analyte, 108EDPQTFYYAVAVVK121, and the stable isotope-labeled internal standard was plotted against concentration. A representative calibration curve is shown in Figure 4S. The LOQ was 500 ng/mL. A representative chromatogram of the LLOQ is shown in Fig. 3A. Because transferrin-free human serum was not available, 5% HSA was utilized as the matrix in this study. As a result, no significant interfering peak was found at the retention time of hTRF in the chromatogram of the blank matrix (Fig. 3B).

The precision and accuracy of the assay were assessed by observing the response of the QC samples with four different concentrations of hTRF in three validation runs. The intra- and inter-day precisions were expressed as the percent coefficient of variation (%CV). The accuracy was obtained by comparing the averaged calculated concentrations to their nominal values (%bias). The results are listed in Table 1. For each QC level of hTRF, the intra- and inter-day precisions were less than 4.9% and 9.0%, respectively.

Table 1
Accuracy and precision for QC samples.

Nominal concentration of hTRF	500 ng/mL	1500 ng/mL	2500 ng/mL	4000 ng/mL
Mean	527	1568	2586	4135
%Bias	5.4	4.5	3.4	3.4
Intra-day precision (%CV)	4.2	3.1	4.9	3.4
Inter-day precision (%CV)	9.0	4.8	3.5	3.8
n	17	18	18	17
Number of runs	3	3	3	3

The bias values were less than 5.4%. Overall, the QC data indicated acceptable accuracy and precision of the current method for the determination of hTRF.

In general, the inefficient tryptic digestion could result in the inaccurate quantification of proteins because the proteolytic peptides, rather than the target protein, were used to prepare the calibration standards [5,23]. However, in this study, the digestion efficiency was not a concern because pure hTRF was employed in the standards. The absolute recovery of hTRF in human serum was calculated by comparing the response ratios of the matrix spiked with hTRF to a neat solution containing the equimolar tryptic peptide. The recoveries of low, mid and high QCs were 87.9%, 87.1% and 82.9%, respectively.

3.4. Method comparison

As previously mentioned, several analytical techniques have been applied to quantify hTRF. To evaluate the LC/MS/MS performance, we compared our results to the values obtained from a conventional immunoturbidimetric method. The regression analysis for the method comparison was also performed using linear regression analysis and Passing–Bablok regression analysis, which was performed using the statistical program Analyze-it® version 2.12 (Analyze-it Software). As shown in Fig. 4A, the LC/MS/MS method was comparable to the immunoturbidimetric assay ($y = 1.194x - 0.457$, $r = 0.9096$, $p < 0.05$), after three volunteers with a low hTRF concentration (<1500 ng/mL) were excluded. The estimated confidence intervals of the slope and the intercept were (0.987–1.40) and (−0.986 to 0.0727), respectively. A Bland–Altman plot (Fig. 4B) was prepared to explore the differences in the analytical procedures for hTRF determination. Their mean difference was −1.03%, where all the individual values were in the range of −11.8% to 8.91%. It should be noted that the concentrations determined from the LC/MS/MS assay for samples 11, 14 and 17 were >10% lower than those measured with the immunoturbidimetric assay and were in the range of 500–1500 ng/mL. Several possible reasons for this discrepancy are incorrect curve-fitting parameters in the chemistry analyzer program or a positive deviation from linearity for the immunoturbidimetric assay at low concentrations (data not shown). In comparison, the LC/MS/MS method provides a lower LOQ (measured as low as ~100 ng/mL) compared to the immunoturbidimetric system. Notably, those volunteers with a low hTRF concentration were among the patients with cirrhosis. This phenomenon has been previously observed [30,31]. Furthermore, compared to concentration of hTRF measured here, the LC/MS/MS approach may be more suitable for the determination of low-abundance proteins in biological matrices. An immunoassay normally requires complex sample pre-treatment and the generation of high-affinity antibodies, and the entire procedure is costly and laborious. Therefore, the establishment of a method for the quantitative measurement of proteins in a complex protein mixture, without a dependency on the generation of antisera or the formulation of an immunoassay, is more applicable for the identification and quantification of novel prospective protein biomarkers. Finally, the proposed approach here is nevertheless interesting for developing quantification methods of other proteins that are not routinely measured by immunoassays (e.g., immunoturbidimetric assays).

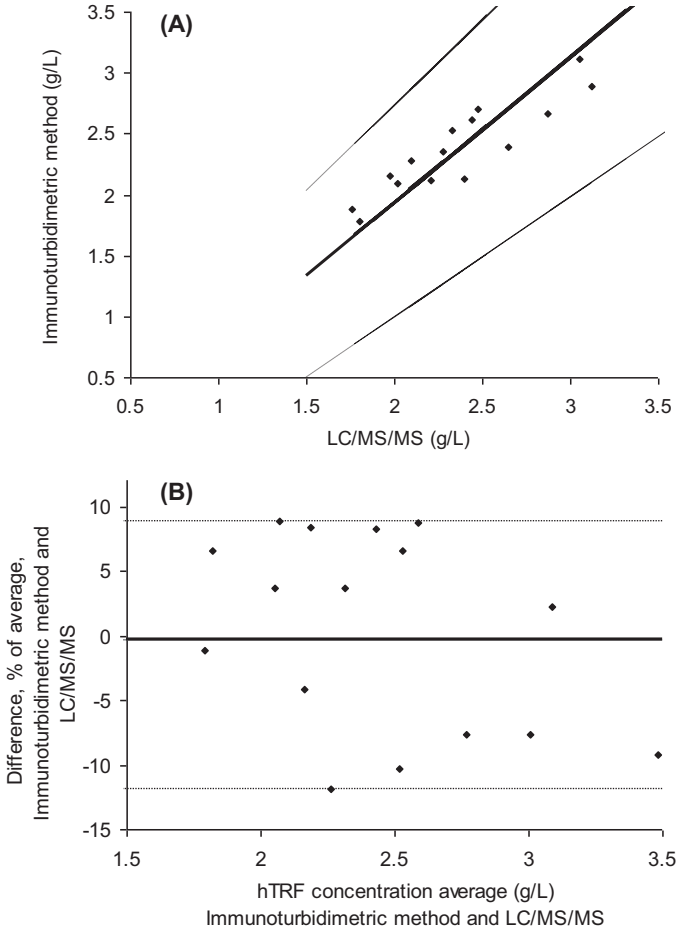

Fig. 4. Passing–Bablok regression analysis (A) and the corresponding Bland–Altman plot (B) for the immunoturbidimetric method vs. the LC/MS/MS assay. The solid line corresponds to the regression line, and the dashed lines represent the 95% confidence interval for the regression line.

4. Conclusions

In this study, a simple and sensitive LC/MS/MS based targeted proteomics assay was developed and validated. Using this assay, hTRF in clinical samples was detected and quantified. Good correlation was observed between this LC/MS/MS assay and an immunoturbidimetric method. These results demonstrated the suitability of the LC/MS/MS assay and its utility to measure differences in the levels of transferrin in various clinical samples. In addition, this targeted proteomics approach provides a lower LOQ and could afford more reliable results, even at low concentrations of hTRF. This report is among the first to demonstrate that the clinical monitoring of hTRF could be achieved by LC/MS/MS. This technology may help provide greater evidence for hTRF as a potential

biomarker for certain cancers. However, a large number of measurements will be needed to further confirm that the LC/MS/MS assay described in this paper is a valuable tool for clinical use.

Ethical approval

This study was approved by the institutional review board of Nanjing Medical University, Nanjing, China.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.006.

References

[1] C.N. Lok, T.T. Loh, Biol. Signals Recept. 7 (1998) 157.
[2] C.P. Bramlage, V.W. Armstrong, A. Zapf, P. Bramlage, G.A. Mueller, M.J. Koziolek, Ther. Apher. Dial. 14 (2010) 136.
[3] J.Q. Sheng, S.R. Li, Z.T. Wu, C.H. Xia, X. Wu, J. Chen, J. Rao, Cancer Epidemiol. Biomarkers Prev. 18 (2009) 2182.
[4] Y.J. Jou, C.D. Lin, C.H. Lai, C.H. Chen, J.Y. Kao, S.Y. Chen, M.H. Tsai, S.H. Huang, C.W. Lin, Anal. Chim. Acta 681 (2010) 41.
[5] D.R. Barnidge, E.A. Dratz, T. Martin, L.E. Bonilla, L.B. Moran, A. Lindall, Anal. Chem. 75 (2003) 445.
[6] J.C. Seegmiller, D. Sviridov, T.S. Larson, T.M. Borland, G.L. Hortin, J.C. Lieske, Clin. Chem. 55 (2009) 1991.
[7] R. Gottschalk, R. Wigand, C.F. Dietrich, G. Oremek, F. Liebisch, D. Hoelzer, J.P. Kaltwasser, Clin. Chim. Acta 293 (2000) 127.
[8] P. Picotti, B. Bodenmiller, L.N. Mueller, B. Domon, R. Aebersold, Cell 138 (2009) 795.
[9] L. Anderson, C.L. Hunter, Mol. Cell. Proteomics 5 (2006) 573.
[10] B. Domon, S. Broder, J. Proteome Res. 3 (2004) 253.
[11] P. Picotti, R. Aebersold, B. Domon, Mol. Cell. Proteomics 6 (2007) 1589.
[12] A. Doerr, Nat. Methods 7 (2010) 34.
[13] A. Doerr, Nat. Methods 8 (2011) 43.
[14] R. Kiyonami, A. Schoen, A. Prakash, S. Peterman, V. Zabrouskov, P. Picotti, R. Aebersold, A. Huhmer, B. Domon, Mol. Cell. Proteomics 10 (2011), M110 002931.
[15] F.R. Food and Drug Administration, Guidance for Industry on Bioanalytical Method Validation, 66(100), 28526 (Docket no. 28598D-21195), Published, 2001.
[16] P. Picotti, O. Rinner, R. Stallmach, F. Dautel, T. Farrah, B. Domon, H. Wenschuh, R. Aebersold, Nat. Methods 7 (2010) 43.
[17] M.B. Strader, D.L. Tabb, W.J. Hervey, C. Pan, G.B. Hurst, Anal. Chem. 78 (2006) 125.
[18] S.K. Wierenga, M.J. Zocher, M.M. Mirus, T.P. Conrads, M.B. Goshe, T.D. Veenstra, Rapid Commun. Mass Spectrom. 16 (2002) 1404.
[19] N. Li, J. Palandra, O.V. Nemirovskiy, Y. Lai, Anal. Chem. 81 (2009) 2251.
[20] Y. Zhang, N. Li, P.W. Brown, J.S. Ozer, Y. Lai, Rapid Commun. Mass Spectrom. 25 (2011) 1715.
[21] P.T. Van, A.K. Schmid, N.L. King, A. Kaur, M. Pan, K. Whitehead, T. Koide, M.T. Facciotti, Y.A. Goo, E.W. Deutsch, D.J. Reiss, P. Mallick, N.S. Baliga, J. Proteome Res. 7 (2008) 3755.
[22] PDB, in http://www.rcsb.org/pdb/explore/explore.do?structureId=2HAU, 2006 (accessed 03.01.12).
[23] O.P. Bondar, D.R. Barnidge, E.W. Klee, B.J. Davis, G.G. Klee, Clin. Chem. 53 (2007) 673.
[24] C.A. Holstein Sherwood, P.R. Gafken, D.B. Martin, J. Proteome Res. 10 (2011) 231.
[25] D.L. Bunker, F.M. Wang, J. Am. Chem. Soc. 99 (1977) 7457.
[26] M.W. Gardner, J.S. Brodbelt, J. Am. Soc. Mass Spectrom. 19 (2008) 344.
[27] Y. Chen, S. Qiao, C. Wang, Int. J. Mass Spectrom. 288 (2009) 68.
[28] Y. Chen, S. Qiao, X. Wang, Y. Liu, Int. J. Mass Spectrom. 295 (2010) 7.
[29] Y. Yu, J. Xu, Y. Liu, S. Qiao, Y. Chen, Clin. Chim. Acta 413 (2012) 894.
[30] B.J. Potter, R.W. Chapman, R.M. Nunes, D. Sorrentino, S. Sherlock, Hepatology 5 (1985) 714.
[31] S. Taguchi, S. Yoshida, Y. Tanaka, S. Hori, Shokuhin Eiseigaku Zasshi 42 (2001) 226.