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A new LC-MS based method to quantitate exogenous recombinant transferrin in cerebrospinal fluid: a potential approach for pharmacokinetic studies of transferrin-based therapeutics in the central nervous system

Shunhai Wang, Cedric E. Bobst, and Igor A. Kaltashov[✉]

Department of Chemistry, University of Massachusetts Amherst, Amherst, MA

Abstract

Transferrin (Tf) is an 80 kDa iron-binding protein which is viewed as a promising drug carrier to target the central nervous system due to its ability to penetrate the blood-brain barrier (BBB). Among the many challenges during the development of Tf-based therapeutics, sensitive and accurate quantitation of the administered Tf in cerebrospinal fluid (CSF) remains particularly difficult due to the presence of abundant endogenous Tf. Herein, we describe the development of a new LC-MS based method for sensitive and accurate quantitation of exogenous recombinant human Tf in rat CSF. By taking advantage of a His-tag present in recombinant Tf and applying Ni affinity purification, the exogenous hTf can be greatly enriched from rat CSF, despite the presence of the abundant endogenous protein. Additionally, we applied a newly developed O¹⁸-labeling technique that can generate internal standards at the protein level, which greatly improved the accuracy and robustness of quantitation. The developed method was investigated for linearity, accuracy, precision and lower limit of quantitation, all of which met the commonly accepted criteria for bioanalytical method validation.

INTRODUCTION

In the past decade, explosive growth in the field of protein therapeutics led to tremendous advances in treating human diseases and has become the fastest growing segment among new pharmaceutical products. This spectacular success is mostly attributed to the unparalleled specificity and activity proteins demonstrate toward their therapeutic targets in comparison to traditional small molecule drugs [1, 2]. However, development of protein drugs targeting pathologies in the central nervous system (CNS) has proved extremely challenging due to multiple obstacles. First, the presence of intracellular and extracellular proteases acts as an enzymatic barrier that can degrade the protein drugs even before they reach the target. Furthermore, the challenge is greatly amplified due to the presence of a formidable physiological barrier between the circulating blood and the interstitial space of the brain, known as the blood-brain barrier (BBB). The BBB consists of tightly linked cerebrovascular endothelial cells that form a diffusional barrier between circulating blood

[✉] address correspondence to: Igor A. Kaltashov, Department of Chemistry, University of Massachusetts-Amherst, 240 Thatcher Drive, Life Science Laboratories #N369, Amherst, MA 01003, Tel: (413) 545-1460, Fax: (413) 545-4490, Kaltashov@chem.umass.edu.

and the interstitial space of the brain. Despite a vast surface area (20 m²) contributing from a massive number of capillaries (100 billion) [3, 4], the brain vasculature is practically inaccessible to over 98% of all small-molecule drugs and all currently approved biopharmaceuticals [5]. To overcome this permeability challenge, tremendous efforts have been devoted to the design of new protein drug molecules that take advantage of endogenous transport systems of the BBB [6, 7].

Transferrin (Tf), an 80 kDa iron transport protein, has attracted much interest as a potential carrier protein for the targeted delivery of various therapeutic agents to the CNS via Tf receptor (TfR) mediated transcytosis (RMT) [8–12]. Due to the high density of TfR on the endothelial cells forming the BBB, Tf possesses a great potential to act as a molecular Trojan horse and ferry tethered drugs across the BBB to the CNS. To study the BBB penetrating ability of exogenous (administered) Tf and/or Tf-based therapeutics, it is critically important to achieve sensitive and accurate quantitation of exogenous Tf in cerebrospinal fluid (CSF). However, this is not a trivial task considering the presence of abundant and structurally similar (if not identical) endogenous Tf in CSF [13], which makes ELISA-based quantitation methods less useful due to cross-reactivity. Radiolabeling can be used as an alternative strategy for tracing exogenous Tf [14], but it requires covalent modification of the protein, which may interfere with its ability to interact with physiological partners and therapeutic targets, thereby affecting its pharmacokinetic and pharmacodynamic properties.

Fortunately, the rapid development of mass spectrometry (MS), particularly in the field of MS-based quantitative proteomics, provides a powerful tool to quantitate protein drugs in circulation with high specificity, accuracy and sensitivity [15, 16]. In order to quantitate a protein drug in complex biological fluids with high sensitivity, an enrichment step is almost always required. Immunocapture is one of the most frequently applied techniques for protein enrichment, which significantly increases the concentration of protein analyte and decreases the complexity of the sample [17–19]. However, this approach is less useful for enrichment of exogenous Tf due to high levels of endogenous Tf in CSF. To overcome this challenge, we took advantage of a His-tag present at the N-terminus of recombinant Tf and applied Ni-NTA (nickel-nitrilotriacetic acid) affinity purification for analyte enrichment. Additionally, to achieve the most accurate protein quantitation by LC-MS, internal standards are frequently required, which can be prepared in various ways [20]. Synthesized stable isotope-labeled peptides are one of the most frequently used internal standards in MS-based protein quantitation [15]. Despite the ease of use, the accuracy and robustness of this approach suffers from an intrinsic limitation. Since the internal standard peptides are combined with the biological samples at the final stage of analysis, variations in upstream sample preparation cannot be corrected for and will ultimately compromise the quantitation results. In particular, peptide-based internal standards cannot compensate for variation in the digestion efficiency of the protein analyte (which frequently does not reach completion and is also affected by miscleavages). Ideally, the stable isotope-labeled protein, produced by using isotopically labeled amino acids in cell culture [21], would be the best internal standard. However, its application is limited due to high costs and difficulties of producing eukaryotic protein standards considering the fact that most protein drugs cannot be expressed in simple bacterial systems [22]. Herein, we applied a cysteine-O¹⁸ labeling

approach recently reported by our group [23] to prepare a Tf internal standard at the protein level. The O^{18} atoms were incorporated into free cysteine residues of Tf by alkylation with O^{18} -labeled iodoacetic acid (IAA), which was prepared in-house by dissolution of IAA in O^{18} -enriched water and incubation at low pH until equilibrium was reached.

Applying the aforementioned techniques, a new LC-MS based approach was developed and validated for the sensitive and accurate quantitation of exogenous Tf (recombinant human Tf, hTf) in rat CSF. We demonstrate that this method has the potential to be utilized for pharmacokinetic studies of administered Tf and/or Tf-based therapeutics targeting the CNS, and can be readily extended to other recombinant proteins incorporating affinity tags as a part of their amino acid sequence.

EXPERIMENTAL

Materials

Recombinant non-glycosylated human serum transferrin (hTf) with 6×His-tag at the N-terminus was provided by Professor Anne B. Mason (University of Vermont College of Medicine, Burlington, VT, USA). Rat cerebrospinal fluid was purchased from Bioreclamation, LLC. (Westbury, NY, USA); Ni-NTA Superflow resin was purchased from Qiagen Science Inc. (Germantown, MD, USA); H_2O^{18} (97% purity), iodoacetic acid (IAA), and mass spectrometry-grade trypsin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The O^{18} -labeled IAA solution (500 mM) was prepared using a protocol reported previously [23]. All other chemicals and solvents used in this work were of analytical grade or higher.

Preparation of calibration standards and quality control (QC) samples

The hTf working solution was prepared by dilution of the stock solution (15 mg/mL) using 100 mM of ammonium bicarbonate to a final concentration of 100 µg/mL. The calibration standards were prepared in triplicates by serial dilutions of the working solution using 10% rat CSF in binding buffer (see below). The concentrations of the calibration standards were 40, 80, 200, 400, 1600, 3200, and 6400 ng/mL, respectively. The QC samples were prepared in five replicates, with the concentrations of 60 (low QC), 1200 (medium QC), and 3600 ng/mL (high QC), respectively. The lower limit of quantitation (LLOQ) standards were prepared at 20 ng/mL based on signal to noise ratios (S/N) observed during method development.

Preparation of internal standards

Internal standards were prepared by diluting 80 µL of hTf working solution (100 µg/mL) into 292 µL of elution buffer (see below). The disulfide bonds in hTf were reduced by adding 8 µL of DTT stock solution (500 mM), and incubated at 50 °C for 30 min. Subsequently, the free cysteine residues were alkylated by adding 20 µL of O^{18} -labeled IAA solution (500 mM), and incubated at 37 °C for 45 min in the dark. The reduced and alkylated hTf internal standard solution (20 µg/mL) was ready to be spiked into calibration standards or QC samples.

Ni-NTA affinity purification of recombinant hTf from spiked rat CSF

A batch processing procedure was applied to enrich recombinant hTf from both calibration standards and QC samples. In a 1.5 mL micro centrifuge tube, 50 μ L of Ni-NTA Superflow resin slurry was washed with 1 mL of binding buffer (50 mM potassium phosphate, 300 mM NaCl, 10 mM imidazole and 10% v/v glycerol at pH 8), centrifuged for 5 min at low speed (5,000xg) to pellet the Ni-NTA resin, and the supernatant was removed. A calibration standard or QC sample was added to the washed resin and binding was conducted at room temperature for 90 min with gentle shaking. Subsequently, the resin was washed using 500 μ L of binding buffer two times to remove non-specifically bound proteins. Finally, hTf was recovered by adding 50 μ L (25 μ L $\times$ 2) of elution buffer (50 mM potassium phosphate, 300 mM NaCl and 300 mM imidazole at pH 7) to the resin and subsequently collecting the supernatant solution. The enriched hTf samples were reduced and alkylated using the same conditions as described above, with the exception that unlabeled IAA was used for alkylation of the reduced cysteine residues. After spiking with 50 ng (2.5 μ L) of internal standard, each sample was digested with trypsin at an enzyme to substrate ratio of 1/100 at 37 °C overnight. The entire workflow is illustrated in Figure 1.

Instrumentation

The digested samples were subjected to LC/MS/MS analysis using an LC Packings Ultimate (Dionex/Thermo Fisher Scientific, Sunnyvale, CA, USA) nano-HPLC system coupled with a QStar-XL (AB SCIEX, Toronto, Canada) hybrid quadrupole/time-of-flight mass spectrometer. A previously reported setup and method were used for the nano-LC/MS/MS analysis [24].

RESULTS AND DISCUSSION

Ni-NTA Affinity Purification

Although CSF is considerably less complex than blood, an enrichment step for protein analyte is still highly preferred considering the fact that the protein drug typically only represents a very small fraction of the total proteome. By introducing a His-tag at the N-terminus of recombinant hTf, the administered NHis-tagged hTf can be effectively enriched from rat CSF by Ni-NTA affinity purification, without the interference from endogenous rat Tf. To demonstrate the necessity of applying Ni-NTA affinity purification, samples containing 1 μ g of NHis-tagged hTf and 50 μ L of rat CSF were digested by trypsin with or without prior Ni-NTA affinity purification step. The digests from each sample were subjected to nano-LC/MS/MS analysis and the results were searched against the SwissProt protein database using the Mascot MS/MS ions search engine [25]. The top five identified proteins are listed in Table 1. Clearly, without the Ni-NTA affinity purification, detection of the relatively high level of NHis-tagged hTf (1 μ g/50 μ L) was unsuccessful due to the abundant endogenous proteins in rat CSF. In contrast, application of the Ni-NTA affinity purification allowed hTf to be readily detected even at a much lower level (0.02 μ g/50 μ L). It is also worth noting that the endogenous rat transferrin was not detected by LC-MS/MS analysis after Ni-NTA affinity purification. This is particularly important if the exogenous protein is structurally very similar or indeed identical to the endogenous one (apart from the His-tag). By removing the endogenous Tf using the Ni-NTA affinity purification step, the

down-stream quantitation can still be performed without interference from the endogenous protein.

Introduction of Internal Standard

Internal standards are critically important in MS-based quantitation in order to achieve the most accurate results, especially when working with complex biological samples where the matrix influence on ionization efficiency of the reference peptide is very significant. Additionally, it is greatly preferred to introduce the internal standards at the earliest stage of the sample preparation workflow so that more sources of quantitation errors can be compensated. By alkylating free cysteine residues in hTf (internal standard) using O¹⁸-labeled IAA, two O¹⁸ atoms were introduced to each cysteine residue at the intact protein level. Although the labeling is limited to cysteine residues, a large number of cysteine-containing tryptic peptides could be generated and utilized for quantitation, as hTf contains 38 cysteine residues. An example of hTf quantitation using this approach is shown in Figure 2. Two tryptic peptides containing either one or two cysteine residues were used as reference peptides to illustrate the quantitation process. For peptides containing one cysteine residue (Figure 2A), an overlap between the two isotope clusters was observed due to the presence of partially labeled internal standard. To minimize this interference, the second isotopic peak from each cluster (I₁ and I₅) was used to calculate the ratio of light (S_L) to heavy (S_H) isotope signal. Additionally, any contribution from a partially labeled internal standard can be corrected for by introducing a labeling coefficient (α); a constant whose value is solely dependent on the purity of the O¹⁸-labeled IAA reagent (the value of α was calculated as the ratio of partially labeled peptide to completely labeled peptide [23]). The following equation was applied to calculate the ratio of peptides from exogenous hTf and internal standard, respectively:

$$\text{peptide ratio} = \frac{S_L}{S_H \cdot (1 + \alpha)},$$
$$\alpha = 0.14$$

For peptides containing two cysteine residues (Figure 2B), the isotopic clusters of peptides from exogenous Tf and internal standard were completely separated. Thus, all the isotopic peaks were combined to generate the extracted ion chromatograms (XICs), and the peak areas were used for calculations:

$$\text{peptide ratio} = \frac{S_L}{S_H}$$

Selection of reference peptide

The primary criterion for selecting a reference peptide for MS-based quantitation is being unique to the analyte (defined by its exact mass, MS/MS identification, and LC-MS retention time), particularly when the protein analyte is also an abundant endogenous protein. Although human Tf and rat Tf only have 73% sequence identity, selective capture of exogenous Tf during the Ni affinity purification step (*vide supra*) allows nearly every Cys-

containing tryptic fragment of hTf to be used for quantitation. Additionally, reference peptides need to be stable during the sample preparation. Thus, peptides that are prone to degradation such as deamidation (e.g. Asn-Gly [26]) and oxidation (Met) should be avoided. Finally, tryptic peptides with missing cleavages are rarely selected as reference peptides, as their abundance can be easily influenced by the digestion conditions. However, a great advantage of using a protein internal standard is being able to eliminate digestion discrepancies. Despite the presence a missing cleavage, peptide DLLFRDDTVCLAK was selected as the reference peptide as it could be consistently detected at very low concentrations. The feasibility of using this peptide for quantitation was further demonstrated by comparing its quantitation results to the results generated for other peptides. As shown in Figure 3, no matter which peptide was used, the quantitation results were well within 20% of the theoretical value. Furthermore, all the peptides displayed a similar trend of variation in quantitation results from three replicate samples. Those results clearly demonstrated that the discrepancy in digestion efficiency between different peptides could be effectively corrected by using protein internal standards, even with peptides containing missing cleavages.

Linearity and Dynamic Range

Using tryptic peptide DLLFRDDTVCLAK as the reference peptide, the measured concentrations were plotted against the theoretical concentrations of hTf in calibration standards (Figure 4), using a $1/\chi^2$ weighted regression. Triplicate sample preparations and measurements were conducted for each data point, and the standard deviation was presented as the error bar. A linear dynamic range of roughly three orders of magnitude was achieved.

Accuracy and Precision

The QC samples were selected to fall within the low, medium and high range of the calibration curve and prepared in replicates of five. Their concentrations were measured using the calibration curve and shown in Table 2. Both the precision (% CV, percent coefficient of variation) and accuracy at all QC levels were well within 15%.

Selectivity and Lower Limit of Quantitation (LLOQ)

The uniqueness of reference peptide DLLFRDDTVCLAK to hTf was confirmed by searching against UniProtKB/Swiss-Prot database using the Protein BLAST search engine. In addition, blank samples containing only rat CSF were processed and analyzed by LC-MS/MS. No interfering signal was observed at the same retention time as the reference peptide. During the method development, the lower limit of quantitation was estimated to be 20 ng/mL based on the S/N ratio (~3). Both the precision (% CV) and accuracy at LLOD level were within 20% (Table 2).

CONCLUSIONS

A new LC-MS based method was developed for sensitive and accurate quantitation of exogenous hTf in rat CSF. By applying Ni-NTA affinity purification, the His-tagged hTf can be greatly enriched from rat CSF, despite the presence of abundant endogenous (rat) transferrin, resulting in a dramatic improvement in sensitivity. Additionally, we introduced

internal standards of hTf at the protein level by utilizing O¹⁸-labeled iodoacetic acid at the alkylation step, which led to more accurate and reliable quantitation. The developed method was investigated for linearity, accuracy, precision and lower limit of quantitation, all of which met the commonly accepted criteria for bioanalytical method validation. This method enables pharmacokinetic studies of Tf-based biopharmaceutical products by providing an attractive solution to a challenging analytical problem of quantitating low-abundance exogenous proteins in complex biological matrices on the background of abundant endogenous proteins which are structurally very similar (or indeed identical) to the administered therapeutic. Additionally, the same methodology could possibly extend to other protein therapeutics with similar analytical challenges under development.

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Figure 1.
Schematic depiction of the sample preparation workflow and introduction of internal standards.

Figure 2.

Examples of protein quantitation using O¹⁸-labeled IAA alkylated hTf as internal standard. (A). using tryptic peptide DLLFRDDTVC*LAK for protein quantitation. (B). using tryptic peptide C*STSSLLEAC*TFR for protein quantitation. The inset figures represent the mass spectra of the Cys-containing peptides generated from O¹⁸-labeled internal standards. The figures on the right represent the extracted ion chromatograms of the selected m/z ranges (grey areas) as shown on the left.

Figure 3.

Comparison of quantitation results using different Cys-containing peptides. The normalized results from triplicate sample preparations and LC-MS analysis were represented with bars in dark gray, gray and light gray (^athe calibrated value was derived from the calibration curve generated by reference peptide DLLFRDDTVC*LAK, Figure 3). Results generated from other peptides were normalized against this value and represented as the height of the bars.

Figure 4.

Calibration curve for quantifying exogenous hTf in rat CSF in the concentration range from 40 ng/mL to 6400 ng/mL constructed using a $1/\chi^2$ regression. The inset shows an enlargement of the lower region of the calibration curve.

Table 1

Shotgun proteomics analysis of rat CSF spiked with exogenous NHis-tagged hTf by Mascot MS/MS Search

A. Without Ni-NTA affinity purification (1.0 µg NHis-tagged hTF in 50 µL CSF)

Protein List	Origin	Mascot Score
Albumin	Rat	1317
Transferrin	Rat	691
Cystatin-C	Rat	166
Clusterin	Rat	165
Hemoglobin	Rat	156

B. With Ni-NTA affinity purification (1.0 µg NHis-tagged hTF in 50 µL CSF)

Protein List	Origin	Mascot Score
Transferrin	Human	1126
Keratin, type II cytoskeletal 1	Human	485
Keratin, type I cytoskeletal 10	Human	223
Trypsin	Pig	218
Keratin, type II cytoskeletal 2	Human	137

C. With Ni-NTA affinity purification (0.02 µg NHis-tagged hTF in 50 µL CSF)

Protein List	Origin	Mascot Score
Haptoglobin	Rat	319
Transthyretin	Rat	302
Transferrin	Human	288
Hemoglobin	Rat	273
Trypsin	Pig	120

Table 2

Accuracy, precision and lower limit of quantitation of the method

QC Samples	Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	number of replicates (n)	CV (%)	accuracy (%)
low QC	60.0	54.2	5	10.2	90.3
Medium QC	1200	1250	5	4.75	104
High QC	3200	3230	5	9.92	101
LLOQ	20.0	16.2	5	10.3	81.2