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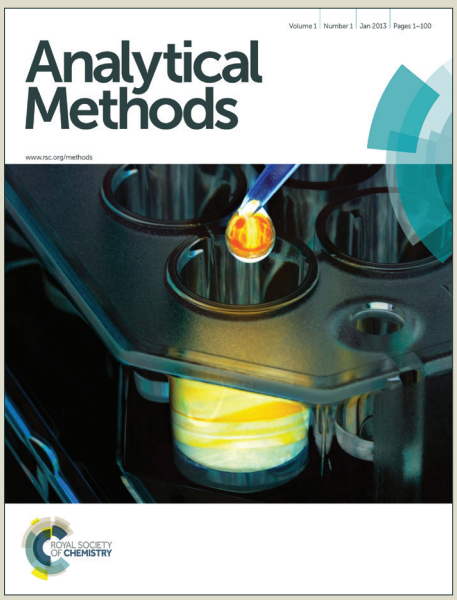

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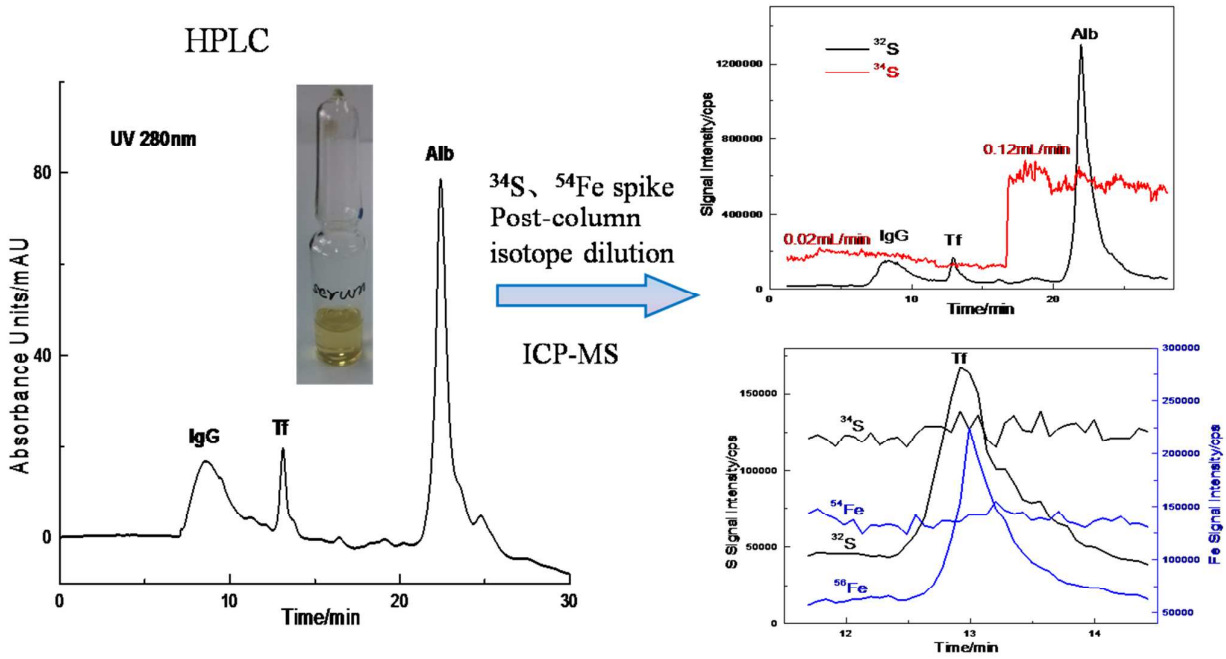
A new simultaneous quantification method for Tf and Alb in human serum via sulfur and iron using HPLC-ID-ICP-MS



Simultaneous Quantification of Proteins in Human Serum via Sulfur and Iron Using HPLC Coupled to Post-column Isotope Dilution Mass Spectrometry

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Abstract:

Quantification of proteins by inductively coupled plasma mass spectrometry (ICP-MS) offers an alternative method for quantitative proteomics. In this study, we developed a method based on high performance liquid chromatography (HPLC) coupled to ICP-MS via post-column isotope dilution for the quantification of transferrin (Tf) and albumin (Alb) in human serum using enriched ^{34}S and ^{54}Fe isotopic solutions. Firstly, Tf, lactoglobulin (Lacb), myoglobin (Myo), and lysozyme (Lys) serving as model proteins were separated with a size exclusion column and permitted the quantification of individual proteins by post-column addition of enriched ^{34}S spike solution. In this way, the methodology was established and validated by comparing with gravimetric results. Then, after Tf saturation and by introducing isotopically enriched ^{34}S and ^{54}Fe spikes at the same time, the human serum Tf was absolutely quantified via both sulfur and iron by means of species-unspecific isotope dilution HPLC-ICP-MS. Good agreement of the two results from sulfur and iron were acquired. Moreover, the concentrations of Tf and Alb in human serum via sulfur using HPLC-ID-ICP-MS were simultaneously determined for the first time by altering the flow rate of enriched ^{34}S isotope solution. All the proposed species-unspecific HPLC-ID-ICP-MS methods were tested for the analysis of a serum certified reference material (ERM-DA470k /IFCC).

Key words: Isotope dilution; HPLC-ICP-MS; Absolute quantification; Protein

1. Introduction

Transferrin (Tf) and albumin (Alb) are of vital importance for pathological state monitoring and clinical diagnosis. Serum Tf is an important beta globulin that adjusts iron ion balance in organisms by means of binding and transporting iron from plasma to cells.[1] In addition, it also acts as bacteriostatic agents and represents an important acute phase protein used for diagnosis and monitoring the treatment of many diseases, such as anemia, inflammation and coronary heart disease.[2,3] On the other hand, human serum Alb is an important protein in human body and the most abundant protein in plasma.[4] Alb acts as one major depot and transport protein for many endogenous and exogenous compounds in the circulation system, regulates the osmotic pressure of plasma, scavenges oxygen free radicals and inhibits platelet aggregation.[5] Moreover, Alb becomes a non-specific indicator for severe illness and is widely used clinically to treat several diseases, such as shock, cerebral edema and hypoalbuminemia.[6] Therefore, the accurate quantification of Tf and Alb in human serum can be of a useful clinical diagnostic tool. The common way to quantify Tf and Alb is by immunonephelometry and immunoturbidimetry. However, the deviation from the overall mean can exceed 15% when comparing different immuno-based quantification methods with each other in inter-laboratory comparisons.[7] For this reason, new accurate quantification methods of Tf and Alb in human serum are urgently needed.

The great potential of inductively coupled plasma-mass spectrometry (ICP-MS) methods and strategies for protein quantification have been highlighted in recent reviews.[8-11] High-performance liquid chromatography (HPLC), with different separating mechanisms, including ion-exchange, reversed-phase, and size-exclusion, is a powerful technique for protein separations in biological samples.[12,13] However, because of the lack of available matrix-matched standards,[14] despite the excellent advantages of HPLC-ICP-MS for elemental quantification in proteins, the validation of the results was still difficult. Unlike external calibration approaches, isotope dilution mass spectrometry method (IDMS), based on the measurement of isotope ratio, is less affected by signal drifts, matrix effects, and analyte losses.[15] Previous studies[16,17] proposed that the application of isotope dilution analysis in HPLC-ICP-MS can be divided into two different modes: species-specific spiking

mode and species-unspecific spiking mode which is also called post-column isotope dilution. Different quantitative strategies of serum Tf based on HPLC-ICP-MS and species-specific or species-unspecific isotope dilution have been developed and the quantification results obtained by species-unspecific isotope dilution are in good agreement with species-specific isotope dilution using synthesized ^{57}Fe-Tf spike.[18, 19] However, preservation of the integrity of metal-protein bond and confirmation of the uniform stoichiometry of metal-protein complex are two prerequisites for accurate quantification of transferrin.[20] Potential metal losses may happen in sample preparation and liquid chromatography separation.[21] Unlike metal elements, sulfur is regarded as the most suitable element for the quantification of proteins which naturally exists in vast majority of proteins, duo to high abundance and covalent bonding in proteins.[22, 23]

Compared to the quantification of Tf, the research on absolute quantification of serum Alb by HPLC-ICP-MS is scarce. For the simultaneous quantification of Tf and Alb in human serum, the species-unspecific isotope dilution method is superior to the species-specific isotope dilution because the isotopically enriched Alb is currently unavailable and the chemical form of spike may be different from the proteins to be determined in species-unspecific IDMS.[24] Also, the main challenge in simultaneous determination of Tf and Alb is the very different concentrations of two proteins in human serum.[25]

In this paper, four standard proteins, including human serum transferrin (Tf), bovine β-lactoglobulin (Lacb), myoglobin in horse (Myo) and lysozyme in chicken (Lys), were selected as model proteins to establish the methodology of absolute quantification of mixed proteins via sulfur by means of species-unspecific isotope dilution HPLC-ICP-MS, and the method was validated by comparing with gravimetric results. Based on this, by introducing isotopically enriched ^{34}S and ^{54}Fe spikes at the same time, the human serum Tf was absolutely quantified via both sulfur and iron by means of species-unspecific isotope dilution HPLC-ICP-MS. Good agreement of the two results were acquired and the human serum certified reference material (ERM-DA470/IFCC) was used for method validation. Moreover, we

developed a method for simultaneous absolute quantification of Tf and Alb in human serum via sulfur element by changing the flow rate of isotopically enriched ^{34}S spike. In this way, the huge sulfur signal intensities discrepancy of the two proteins could be resolved and more accurate results were achieved.

2. Experimental Section

Reagents, Proteins, and Standards

Tris(hydroxymethyl)-aminomethane, Tf, Alb, Lys，and Lacb were purchased from Sigma (St. Louis, MO, USA). The number of sulfur atoms and purity of each protein were shown in Table 1. Ammonium formate, ammonium acetate, acetic acid, and methanol were purchased from Fisher Scientific (USA). The human serum certified reference material (ERM-DA470/IFCC) was purchased from JRCs Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Human serum sample from healthy volunteers and Myo were provided by National Institute of Metrology (NIM, Beijing, China).

The isotopically enriched ^{54}Fe spike solution was provided by National Institute of Metrology (Beijing, China). The certified concentration of isotope-enriched ^{54}Fe spike was 8.8836 $\mu g \cdot g^{-1}$ and the certified ratio of $^{56}Fe/^{54}Fe$ of the solution was 0.11313. The enriched ^{34}S powder was from Cambridge Isotope Laboratories (USA), which had the 99.99% purity. The solution of isotope-enriched ^{34}S spike was prepared by deionized water (18 MΩ·cm, Millipore, USA). The concentration of isotope-enriched ^{34}S spike was 5.594 $\mu g \cdot g^{-1}$ and the isotopic ratio of $^{32}S/^{34}S$ of the solution was 0.01417, which was measured by TIMS (Isoprobe, GV Instruments). The concentration of spike solution was gravimetrically diluted so as to obtain an optimal isotope ratio in the mixture of sample and isotope-enriched spike. For instrumental mass bias correction, the sulfur isotopic reference material with natural abundance (IRMM-644) was from IRMM and iron isotopic reference material with natural abundance (GBW08446) was from NIM, China.

Table 1 Number of sulfur atoms and purity of the proteins

Proteins	Number of S atoms in each molecular	Purity
Tf	47	⩾98%
Lacb	9	⩾90%
Myo	3	⩾90%
Lys	12	⩾98%
Alb	41	⩾98%

Instrumentation

HPLC. The HPLC system consisted of a LC-30AD pump, a SIL-20A automatic sampler, and a SPD-20A UV-detector (Shimadzu, Japan). The wavelength of 280nm was chosen to monitor the protein. A size exclusion column TSK-gel G3000SWxl column (7.8mm×300mm) with a mass range of 10-500 KDa was used for four standard proteins separation. The SEC mobile phase was 100 mM ($mmol \cdot kg^{-1}$) ammonium acetate and the chromatographic condition was 0.4$mL \cdot min^{-1}$ isocratic elution for 40min. A strong anion-exchange column Shodex QA-825 (8.0mm×75mm) was used for protein separation of human serum. The mobile phase consisted of a gradient of ammonium formate (0-500 mM) in 20 mM Tris-HAc, pH= 8.6. The flow rate of the mobile phase was 0.7 $mL \cdot min^{-1}$, and the chromatographic time was 30min.

HR-ICP-MS. The sector field ICP-MS was Element 2 from Thermo Fisher Scientific (Bremen, Germany), which was operated at medium resolution ($m/\Delta m$=4000) through all the experiments. The optimum instrumental parameters used on sector field ICP-MS and HPLC were given in Table 2. A tuning solution (1 $ng \cdot g^{-1}$ Be, In, Bi) was used in the HR-ICP-MS tuning process. The dead time of the HR-ICP-MS detector was calculated as 18 ns. And then the dead time was introduced in the software of the HR-ICP-MS for automatic correction. For the Element 2 ICP-MS, the scope of counting mode can reach 3~4 million cps. In this experiment, the maximum cps of ^{32}S was about 1.2 million. Therefore, both ^{32}S and ^{34}S were determined by the counting mode. Moreover, in the method edition, the detection mode was set as “Both” in the determination of ^{32}S and ^{34}S, in this way, the instrument would carry out the counting-analog calibration automatically. A Toledo electronic balance (Mettler

Company, Germany) was used to accurately weigh the mass of proteins.

Table 2 Instrumental operating condition for HPLC and ICP-MS

HPLC Parameters		
Column	TSK-gel G3000SWxl(7.8mm×300mm)	Shodex QA-825(8.0mm×75mm)
Mobile phase	100 mM ammonium acetate	(A) 20 mM Tris-HAc, pH= 8.6 (B)A+500 mM ammonium formate
Injection volume	20μL	10μL
Gradient	isocratic elution for 40min	0~100% B in 30min
Flow rate	0.4mL·min^{-1}	0.7mL·min^{-1}
Detection wavelength	UV at 280nm, 460nm	UV at 280nm
ICP-MS Parameters		
RF power	1233W	
Cooling gas flow rate	16.00 L·min^{-1}	
Auxiliary gas flow rate	0.96 L·min^{-1}	
Sample gas flow rate	1.11 L·min^{-1}	
Resolution	m/Δm=4000	
Isotopes monitored	^{13}C,^{32}S,^{34}S,^{54}Fe,^{56}Fe	
Runs and Passes	700×1	
Replicate measurements	3	

Sample preparation

The mixed aqueous solution of four standard proteins, including Tf, Lacb, Myo, and Lys was filtered through 0.22 μm syringe filters and then injected into the HPLC system (20μL).

The human serum protein reference material ERM-DA470/IFCC was reconstituted according to the protocol provided by the manufacturer. Since the quantification methods of Tf required an uniform iron stoichiometry (two iron ions per Tf molecule), 300 μL of a 25 mM Tris-HAc buffer was combined with 5 μL of 500 mM Na_2CO_3 solution, and 5 μL of 10 mM $FeCl_3$ solution and 100 μL of serum sample were kept for 60 min at room temperature.[26] The sample solutions were then filtered through 0.22 μm syringe filters. All samples were kept in a refrigerator at 4℃ until measurement.

Species-unspecific isotope dilution analysis

Size exclusion chromatography (SEC) based on the different molecular weight was used to separate the four standard proteins. Tf and Alb in human serum were

separated by strong anion exchange chromatography as the molecular weight of the two proteins was similar which results in an inadequate separation in SEC column. The protein peaks were identified by retention time matching with standard proteins.

For species-unspecific isotope dilution analysis of the mixed standard proteins, the enriched isotope solution containing 3.135 μg·g^{-1} ^{34}S was continuously added into the eluate by a LC-20AD pump (at 0.4 mL·min^{-1}) at the end of the SEC column through a Y-formed three-way connection. When the sample was human serum, the mixed enriched isotope solution of 5.594 μg·g^{-1} ^{34}S and 0.401 μg·g^{-1} ^{54}Fe was prepared in 25 mM Tris-HAc buffer and continuously introduced into the eluent coming from the strong anion exchange column in the same way. Moreover, in consideration of the significant difference in content of Tf and Alb in human serum, the flow rate of spike solution was changed over elution time. In the first 15 minutes (Tf elution), the flow rate of enriched isotope ^{34}S、^{54}Fe was 0.02 mL·min^{-1}, then the flow rate changed to 0.12 mL·min^{-1} for the rest time (Alb elution). Because of the similar performance of separating pump (LC-30AD) and spiking pump (LC-20AD), the signal intensity for ^{34}S、^{54}Fe changed quickly and remained stable when the flow rate of spike solution altered. All connections were made of PEEK tubings with the diameter of 0.13 mm diameter.

In addition, a sector field ICP-MS was used at medium resolution (m/Δm=4000) to separate polyatomic interferences in order to monitor isotopes of S and Fe. In the determination of ^{32}S/^{34}S and ^{56}Fe/^{54}Fe, the mass bias corrections were conducted by the using the sulfur and iron isotopic CRM solution with natural abundance (IRMM-644 and GBW08616). Considering the variance of ^{32}S/^{34}S in the unspiked protein sample, the ^{32}S/^{34}S ratios of Tf and Alb were determined by HR-ICP-MS without HPLC. The Tf and Alb samples were dissolved with water separately, and the ^{32}S/^{34}S ratio of each protein solution was determined with the IRMM-644 solution for instrumental mass bias correction. The intensity chromatograms (cps·min^{-1}) were transformed into the mass flow chromatograms (ng·min^{-1}) using the on-line isotope dilution equation after mathematical treatments. The amount of S and Fe in proteins was obtained by integration of corresponding chromatographic peaks using Origin 8.0

(OriginLab Corporation, USA). What is more, the concentration of each protein was calculated by the absolute amount and the stoichiometric ratio of S and Fe in proteins.

3. Results and Discussion

Absolute quantification of mixed standard proteins via sulfur by post-column HPLC-ID-ICP-MS.

Size exclusion chromatography (SEC) was always used to separate mixed proteins with different molecular weight. Ammonium acetate buffer was used as mobile phase for the separation of four proteins (Tf, Lacb, Lys, and Myo). Instead of the usually used sodium chloride in phosphate buffer, ammonium acetate buffer was used to avoid salt deposition on the cones of ICP-MS.[27] In this study, the optimal ammonium acetate concentration was investigated to get good separation efficiency. Ammonium acetate concentration ranged from 50 to 200 mM. When the concentration of ammonium acetate decreased to 50 mM, only three proteins could be eluted in liquid chromatography. And the peak shape improved little when the concentration increased to 200 mM. Therefore, 100 mM ammonium acetate was chosen to guarantee the complete separation of four proteins and reduce carbon deposition on the ICP-MS cones. As shown in Fig 1, it can be seen that four standard proteins were separated successfully in 40 min, and the retention time of Tf, Lacb, Myo, and Lys were 21.6, 23.5, 26.5, and 32.6 min respectively.

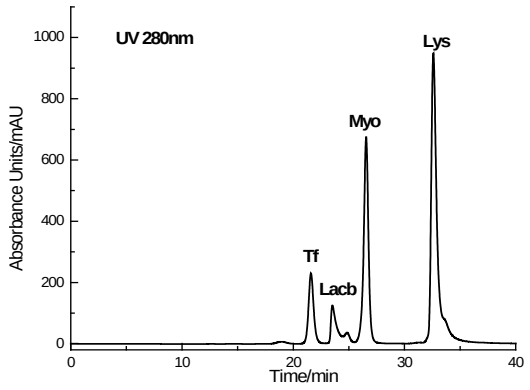

Fig. 1 Liquid chromatography of four standard proteins

Following the separation of the four standard proteins by SEC, the enriched ^{34}S isotope solution was continuously mixed with the eluate from the HPLC via a three-way connection. The isotope ratio $^{32}S/^{34}S$ was measured online by HR-ICP-MS

at medium resolution. The contents of sulfur in each protein were calculated by post-column isotope dilution equation which was well summarized in the literature.[16,28,29] In brief, the chromatogram of isotope ratio was transformed into the chromatogram of mass flow (mass vs time) using the following isotope dilution equation:

$$MF_s = c_{sp} d_{sp} f_{sp} \frac{M_s}{M_{sp}} \frac{A_{sp}^b}{A_s^b} \frac{R_m - R_{sp}}{R_s - R_m}$$

where MF_s has units of ng·min^{-1} and it is the mass flow of sulfur in the sample eluting from the column, c_{sp} is the concentration of the element sulfur in the spike (ng·g^{-1}), $d_{sp}f_{sp}$ has units of g·min^{-1} and it is the mass flow rate of the spike. Also, M_{sp} and M_s are the atomic weights of the element sulfur in the spike and the sample, respectively. A_{sp} and A_s are the abundance of the isotope "b"(^{34}S) in the spike and the sample. R_m, R_{sp}, and R_s are the isotope ratios (isotope a/isotope b, ^{32}S/^{34}S) in the mixture, spike, and the sample, respectively.

In the above isotope dilution equation, the mass flow ($d_{sp}f_{sp}$) of this work was assessed by gravimetric data. It was determined as follows: the mass flow of spike solution transmitted by spiking pump was weighed at a certain flow rate (such as 0.4 mL·min^{-1}) for 1 min. 7 replicates were conducted to calculate the average $d_{sp}f_{sp}$. The integration of corresponding protein peaks in the mass flow chromatogram will obtain the absolute amount of sulfur in that protein. According to the volume injection and the sulfur stoichiometric ratio in each protein, the concentration of proteins can be calculated.

The isotope ratio of ^{32}S/^{34}S measured by ICP-MS and the mass flow of sulfur were illustrated in Fig 2. The only measured parameter was the isotope ratio R_m in the mixture, which was less affected by matrix effects in mobile phase and the instrument drift. In Table 3, it showed that the concentrations of the four proteins obtained via sulfur by post-column isotope dilution analysis were in good agreement with the gravimetric results, which validated the established methodology. The relative standard deviations (RSDs) in the experiments were less than 5% for each protein, which was better than the isotope coded affinity tags (ICAT) technique with the RSD around 4~28%.[30]

Table 3 Absolute quantitative analysis of proteins (n=3)

Proteins	Absolute quantity of sulfur (ng)	Protein concentration (mg • mL^{-1})	
		Isotope dilution analysis	Gravimetric method
Tf	667±11	1.66±0.03	1.69
Lacb	429±7	1.36±0.02	1.38
Myo	397±15	3.52±0.13	3.52
Lys	1630±14	3.03±0.03	3.15

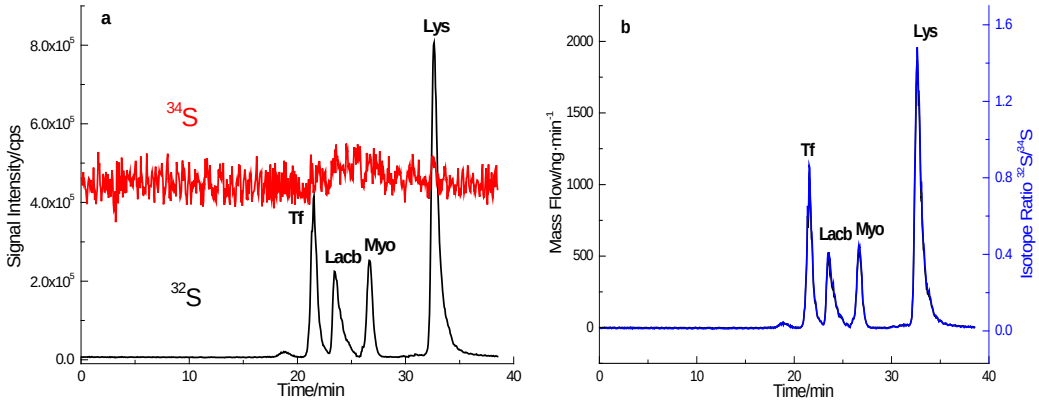

Fig. 2 Mass spectrum of mixed standard proteins, a: HPLC-ID-ICP-MS; b: chromatogram of mass flow of sulfur and isotope ratio

Study of the iron saturation grade in Tf by HPLC

Commonly, the degree of iron saturation in Tf was investigated by ultraviolet-visible spectrophotometry through the ratio of absorbance at 280 nm (total amount of protein) and 460 nm (the binding of Fe to tyrosine). For holo-Tf, the ratio was around 22.[31, 32] As UV-vis detector in HPLC had the similar principle with ultraviolet-visible spectrophotometry, we attempted to investigate the saturation grade of Tf using UV-vis detector of HPLC. Tf-saturated and Tf-blk (the same procedure as iron saturation without 5μL $FeCl_3$ solution) were separated by TSK-gel G3000SWxl column and detected at 280 nm and 460 nm respectively (Fig. 3). Then the efficiency of iron saturation was evaluated through the ratio of absorbance value at 280nm and 460nm.

As shown in Fig 3, the retention time of Tf with or without saturation process at 280 nm and 460 nm had no difference and were both at 21.5 min. What is more, the absorbance value (mAU) in Fig 3 showed that the ratio of Tf without saturation (Tf-blk) at 280nm and 460nm was 33.6. However, the ratio of saturated Tf

(tf-saturated) was 22.1, which was close to 22 in holo-tf. The obtained results indicated that the iron saturation of Tf was successful and the same procedure was used to quantify Tf in human serum and ERM-DA470/IFCC sample.

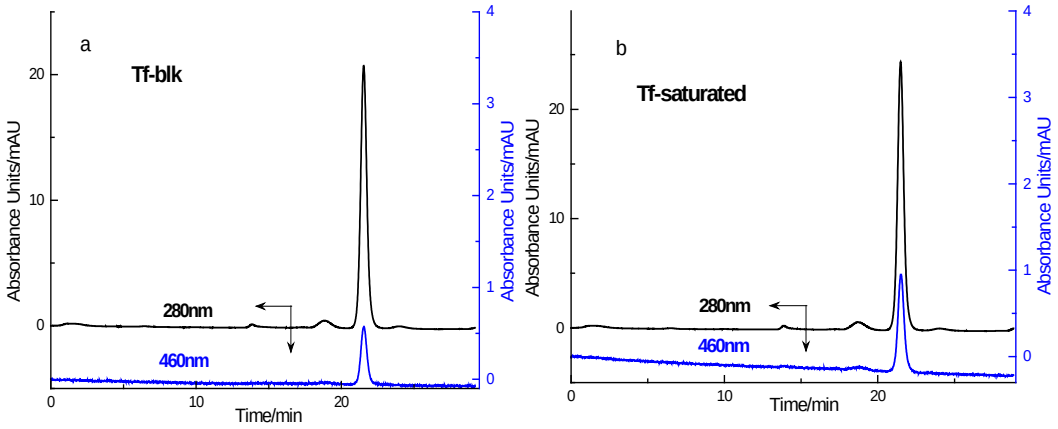

Fig. 3 Liquid chromatography of Tf blk (a) and Tf with saturation (b) using UV detection of HPLC at 280nm and 460nm

Absolute quantitative analysis of Tf via iron and sulfur in human serum by post-column HPLC-ID-ICP-MS.

As the molecular weight of Tf was close to Alb, the retention time of the two proteins was overlapped in SEC and could not be entirely separated.[33] Therefore, anion exchange column (Shodex QA-825) was used to separate Tf and Alb in human serum. The separation condition was a linear gradient of 30 min from 0 to 100% of buffer B (A+500 mM ammonium formate) in 20 mM Tris-HAc as buffer A. The liquid chromatogram in human serum with UV detection (at 280nm) was shown in Fig 4. It can be seen that immunoglobulin (IgG) was first eluted at 8.3 min, and then Tf was at 13.0 min, followed by Alb at 21.8 min. The chromatography baseline was not very stable, which may be associated with the constantly changing mobile phase in gradient elution.[20]

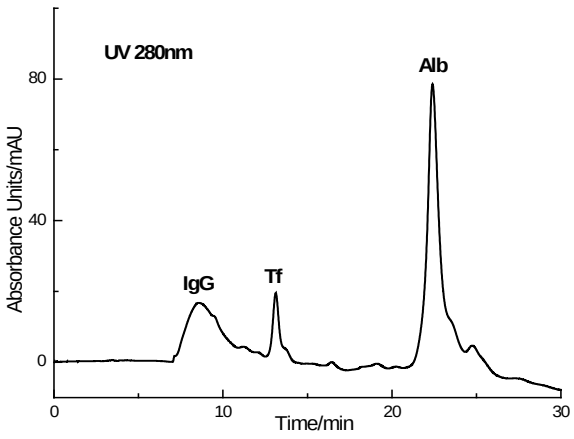

Fig. 4 Liquid chromatogram of human serum

In this experiment, the Tf in ERM-DA470/IFCC and human serum sample was analyzed. The enriched ^{34}S and ^{54}Fe spike solutions were added into the elute at the end of column. The ratio of $^{32}S/^{34}S$ and $^{56}Fe/^{54}Fe$ were obtained from the integration of intensities of ^{32}S, ^{34}S, ^{54}Fe and ^{56}Fe in the chromatographic procedure of Tf (shown in Fig 5), and then the absolute amount of sulfur and iron could be calculated by the on-line HPLC-ICP-MS equation. Since the number of sulfur and iron atoms in Tf were known, the concentrations of Tf in ERM-DA470/IFCC and human serum sample could be calculated by both sulfur and iron (in Table 4).

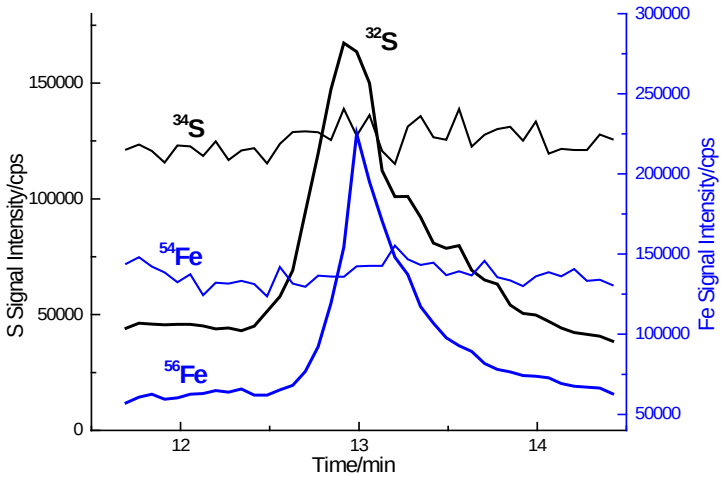

Fig. 5 The intensity of ^{32}S, ^{34}S, ^{54}Fe, and ^{56}Fe in Tf chromatographic procedure

Table 4 Absolute quantitative analysis of Tf in ERM-DA470/IFCC and human serum sample via iron and sulfur (n=3)

Quantitative element	Tf in CRM ($mg \cdot mL^{-1}$)	*Certified value ($mg \cdot mL^{-1}$)	Tf in human serum ($mg \cdot mL^{-1}$)
Iron	2.39±0.20	2.32±0.08	2.50±0.11
Sulfur	2.36±0.04		2.49±0.02

*: Certified value of Tf in ERM-DA470/IFCC

As can be seen in Table 4, the concentrations of Tf determined by the proposed species-unspecific ID-MS method via iron and sulfur were in good agreement of both the ERM-DA470/IFCC and human serum sample. Moreover, the measured Tf value of ERM-DA470/IFCC was consistent with the certified value, which proved the validity of the method. The precision and RSDs obtained by sulfur were better than by iron. This phenomenon might be related to the different existing forms of iron and sulfur. Sulfur always exists in the form of covalent bond in cysteine or methionine with good stability and wide abundance in proteins, while the binding between iron and protein is non-covalent that the force is weak and more susceptible to external conditions, such as the pH of mobile phase, ionic strength, and other polar solvents. This is also the reason that species-specific spikes available are mainly metalloproteins, and it is almost impossible to exchange sulfur isotope in vitro and develop the sulfur enriched species-specific spike in spite of sulfur existing abundantly in proteins.[34]

Simultaneous quantification of Tf and Alb via sulfur in human serum by species-unspecific HPLC-ID-ICP-MS.

Sulfur(S) is the most suitable element for quantitative analysis of proteins in virtue of its widely distribution and stability in proteins. As we know, there is significant difference in the content of Tf and Alb in human serum, which results in great disparity in sulfur content of different proteins. Therefore, in the simultaneous species-unspecific analysis of Tf and Alb, the choice of optimal $^{32}S/^{34}S$ ratio for both Tf and Alb was especially challenging. In this study, the ^{34}S spike solution with constant concentration was introduced by LC-20AD pump, and the flow rate of

post-column spike was altered to adjust the optimal $^{32}S/^{34}S$ ratio in the elution procedure. The flow rate of enriched ^{34}S spike solution was set at 0.02 mL·min^{-1} at the first 15 min (Tf elution), and increased to 0.12 mL·min^{-1} to fit for the tremendous difference of Alb sulfur content, and the signal can be changed and achieved stable quickly when the flow rate was altered. Also, the method was validated by ERM-DA470/IFCC and good agreement with the certified value was achieved.

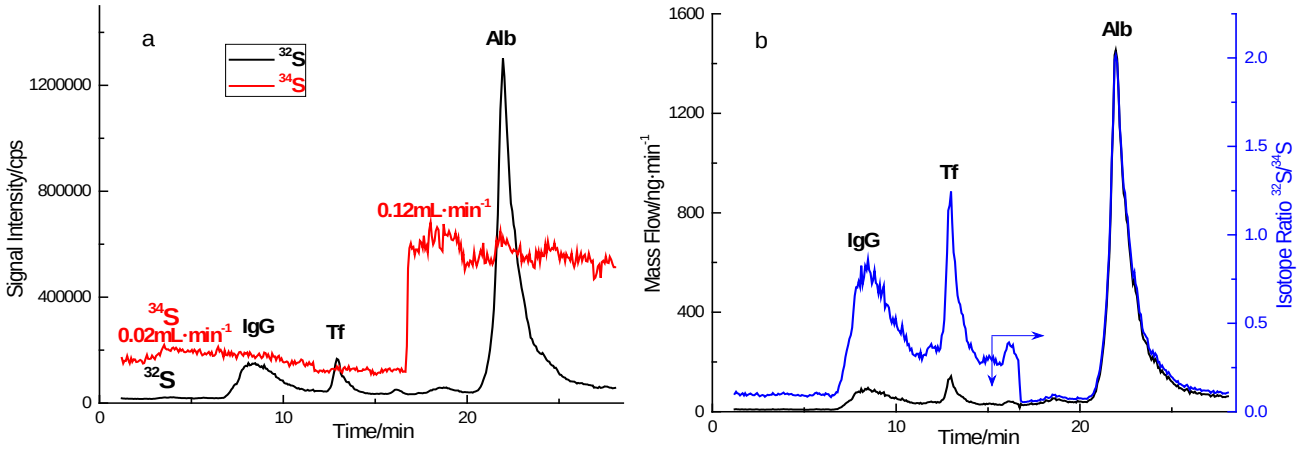

Fig 6 Mass spectrum of human serum, a: HPLC-ID-ICP-MS; b: chromatogram of mass flow of sulfur and isotope ratio

Table 5 Absolute quantitative analysis of Tf and Alb in ERM-DA470/IFCC and human serum sample (n=3)

Proteins	Concentrations in CRM (mg·mL^{-1})	*Certified value (mg·mL^{-1})	Concentrations in human serum (mg·mL^{-1})
Tf	2.36±0.04	2.32±0.08	2.49±0.02
Alb	36.9±0.7	36.5±1.1	40.1±0.6

*: Certified value of Tf and Alb in ERM-DA470/IFCC

The chromatogram of isotope ratio was transformed into mass flow chromatogram using post-column isotope dilution equation (in Fig 6), and the absolute amounts of sulfur in Tf and Alb were obtained by integration the corresponding peaks. The concentrations of Tf and Alb in ERM-DA470/IFCC and human serum sample (in Table 5) were calculated by the sulfur stoichiometry in proteins and the injection volume. As shown in Table 5, the results of Tf and Alb in ERM-DA470/IFCC were in good agreement with the certified value, which validated the established methodology of HPLC-ID-ICP-MS again. Also, the measurement contents of Tf and Alb in human serum were within normal concentration range. Moreover, the method for

quantification of other substrate proteins in serum was also feasible and accurate.

4. Conclusions

Accurate quantification methods of proteins in matrix sample are urgently needed. In this study, the methodology based on species-unspecific isotope dilution HPLC-ICP-MS had been established for the quantification of several proteins in human serum. First, four standard proteins served as model proteins were used to develop and validate the method by comparing the quantitative analysis with gravimetric results. Additionally, by synchronously introducing the ^{54}Fe and ^{34}S spike solution after the column, the content of Tf was absolutely quantified via both iron and sulfur simultaneously. The measured results of Tf in ERM-DA470/IFCC via sulfur and iron were 2.36 ± 0.04 mg·mL^{-1} and 2.39 ± 0.20 mg·mL^{-1} respectively, which coincided with the certified value 2.32 ± 0.08 mg·mL^{-1}. Moreover, the RSDs obtained by sulfur was better than iron. Finally, the concentrations of Tf and Alb in human serum via sulfur using HPLC-ID-ICP-MS were simultaneously determined the first time by altering the flow rate of enriched ^{34}S isotope solution. The quantitative results of ERM-DA470/IFCC were in good agreement with the certified values and the content of proteins in human serum sample was within normal concentration range.

The study provided a precise and reliable way to use HPLC-ID-ICP-MS technique for the absolute quantification of standard and matrix proteins. The application to quantitative analysis of human serum revealed the enormous potential of inorganic mass spectrometry (ICP-MS) coupled techniques to tackle the challenging quantitative proteomics.

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