Analytical Methods

PAPER

Quantification of total serum transferrin and transferrin sialoforms in human serum; an alternative method for the determination of carbohydrate-deficient transferrin in clinical samples†

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Carbohydrate deficient transferrin (CDT) is a biochemical marker for congenital disorders of glycosylation (CDG), chronic alcohol consumption, and forensic medicine diagnosis. However it is necessary to take into account that CDT is not a single molecular entity but refers to a group of transferrin (Tf) sialoforms (asialo-, monosialo-, disialo- and occasionally trisialo-Tf). A number of methods have been developed for CDT measurement based on different analytical techniques and principles without harmonization or calibration to a reference method or a certified reference material, hampering understanding of the diagnostic value of CDT and its routine use. Thus, it is unquestionable that there is a need for a reference material which permits the accurate and precise determination of each individual Tf sialoform which could serve as a universal calibrator for routine immunologic methods used in clinical laboratories. In this work, we describe highly sensitive ICP-MS isotope dilution analysis (IDA) methods for the separation and quantification of the different Tf sialoforms in human serum. The methodology was applied to measure the concentration of each sialoform of Tf and the total concentration of Tf in the NIST Standard Reference Material (SRM) 909c human serum. Additionally, two clinical laboratory control serums utilized for routine analysis of CDT were also analyzed. The separation of the sialoforms was achieved by anion exchange chromatography. The two IDA techniques applied for the quantification of the Tf sialoforms were: (a) post-column IDA and (b) species-specific IDA with the total concentration of Tf calculated by these two being the sum of the individual sialoforms. A third IDA technique, exact matching IDA was applied to determine the total concentration of Tf in SRM 909c. All the Tf measurements were validated with the ERM-DA470-IFF human serum (IRMM, Geel, Belgium) certified for total Tf. Finally, the identification of each Tf sialoform previously separated in the serum (SRM 909c) was carried out by LC/MS/MS.

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Introduction

Tf is the most important iron-transport glycoprotein with a M_w of 79 573 Da and consists of a single polypeptide chain of 679 amino acids, two independent metal iron-binding sites (one within the N-terminal and the other within the C-terminal lobe) and two N-linked complex glycan chains. The two N-linked glycans differ in their degree of branching (with each showing bi, tri, and tetra-antennary structures) and each antenna terminated in a negatively charged sialic acid (*N*-acetylneuraminic acid) molecule. Given the differences of the sialic acid content of the two Tf *N*-glycan chains, a total of nine different Tf isoforms or sialoforms can be distinguished. The most abundant Tf sialoform under normal conditions contains two disialylated biantennary glycans (*i.e.*, a total of four terminal sialic acid residues) named tetrasialotransferrin (tetrasialo-Tf), which totals between 64 and 80% of total serum Tf.[1] Less abundant sialoforms also found in healthy individuals have two (disialo-Tf, approximately 1.0–1.5%), three (trisialo-Tf, approx. 4–5%), five (pentasialo-Tf, approx. 15%), six (hexasialo-Tf, approx. 1%) and seven (heptasialo-Tf, <1.5%) sialic acid residues.[2,3]

The Tf molecules' pI decreases by ≈0.2 pH units with each Fe^{3+} ion bound and by ≈0.1 pH units with each sialic acid residue bound to the *N*-glycan chains,[4] demonstrating the distinct microheterogeneity of human serum Tf. To reduce the number of Tf isoforms occurring in the native serum sample with equal pI, the sample preparation requires *in vitro* Fe^{3+} saturation of the Tf (approx. 30% saturated in healthy

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individuals). This treatment establishes a uniform Tf iron load allowing separation of the isoforms depending solely on the sialic acid content with the sialoforms of lower sialic acid content (asialo-, monosialo- and disialo-Tf) referred to as CDT.[5,6] The measurement of CDT is a biochemical marker of moderate to heavy alcohol consumption, unfortunately, there is no universal definition of the measurand of CDT.[7] This fact prompted the initiation of the Working Group on CDT Standardization (WG-CDT) under the auspices of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), who have proposed disialo-Tf as the primary target analyte for CDT standardization[8] and is reported as relative % CDT (disialo-Tf/Σ of sialoforms $\times$ 100).

CDT measurements in human serum constitute the most specific marker for chronic heavy alcohol use and CDG in proteins.[8] Therefore, the development of sensitive, precise, and accurate analytical methods for individual CDT determination is of significant importance because reliable CDT standards are, so far, not commercially available. CDT was originally separated, identified and quantified by isoelectric focusing (IEF),[6] often coupled to an immunometric recognition step.[5,9] This analytical approach, although being highly selective, shows high inaccuracy and imprecision of the quantitative evaluation, based on off-line staining and densitometry. For this reason, it has been progressively substituted by more quantitatively accurate techniques, such as liquid chromatography (LC),[7,10,11] capillary electrophoresis (CE),[12–14] N Latex CDT immunnoassay[15] and more recently, sophisticated mass spectrometric methods.[16,17] These methods differ in that they can include various sialoforms of asialo-Tf, monoasialo-Tf, disialo-Tf and/or trisialo-Tf in the clinically reported "CDT" fraction.[9] This has been shown to be disadvantageous for CDT testing because of the propensity for the inclusion of variable proportions of trisialo-Tf in the CDT fraction,[9,17,18] which produces falsely high and inconstant CDT results.[19–21]

At present, the LC-UV/vis technique is the recommended reference method of the WG-CDT, until a suitable mass spectrometric method can be established.[8] Standardization for routine clinical measurements of % CDT amongst different methods and even amongst different laboratories running the proposed WG-CDT reference method has been an ongoing source of interest and the main limitation in CDT analysis.[22] This lack of standardization complicates the preanalysis, analysis and interpretation of CDT which also hampers clinical and analytical comparison between clinical studies using different measurement methods.

In this work we propose a more selective, sensitive and accurate method for the determination of Tf isoforms based on the coupling of the LC to a SF-ICP-MS with the aim of producing a SI traceable reference material for clinical CDT measurements. The main advantage of using ICP-MS detection, apart from the increased specificity and sensitivity, is the possibility of conducting quantitative elemental and isotopic analysis. Among the different metal quantification methods that can be conducted by ICP-MS, IDA techniques provide the best capabilities in terms of both precision and accuracy. Depending on when and in which chemical form the isotope tracer (spike) is added to the sample, the application of isotope dilution analysis for elemental speciation can be performed under two different modes (the so-called "species-unspecific" and "species-specific" modes).[23]

Materials and methods

Standards, reagents and samples

All chemicals and reagents used in this work were of analytical grade. Human serum Apo-Tf standard was purchased from Sigma Aldrich (St. Louis, MO, USA). Locally prepared Milli-Q (18.2 mΩ cm) water was used as a diluent to prepare all solutions. Tris-hydroxymethyl-aminomethane (Tris), ammonium bicarbonate, sodium chloride, ammonium acetate and sodium citrate were purchased from Sigma Aldrich. The ^{57}Fe isotopic standard (96.06% enriched) (lot # U11852) was purchased from Trace Sciences International (Richmond Hill, Ontario, Canada). SRM 3126a Iron Standard Solution (lot # 051031) and SRM 909c human serum (fresh-frozen pooled serum) were obtained from NIST (Gaithersburg, Maryland, USA). All the solutions were then prepared from the corresponding stock solutions by dilution with Milli-Q water and acidified with optima grade nitric acid (HNO_3) purchased from Thermo Fisher Scientific (Hudson, New Hampshire, USA). A low serum pool with % CDT in the normal range for control individuals and high serum pool with higher % CDT, typically from heavy alcohol consumers, were obtained from the Medical University of South Carolina's (MUSC) Clinical Neurobiology Laboratory and had been assayed by the IFCC's WG-CDT recommend reference LC method which had previously been calibrated with the IFCC workgroup labs. ERM-DA470-IFF human serum (IRMM, Geel, Belgium) with a SI traceable certified mass fraction of Tf (2.36 $\pm$ 0.08 g L^{-1}) was utilized as a control material for the total Tf measurements.

Instrumentation

LC/SF-ISP-MS. The chromatographic separation was accomplished on a Dionex ICS-3000 Dual pump (Sunnydale, CA, USA). The anion exchange column used was a Dionex ProPac SAX-10 (4 mm $\times$ 250 mm). Mobile phase A consisted of Tris–HCl 10 mmol L^{-1} pH 7 and mobile phase B and C consisted of Tris–HCl 50 mmol L^{-1} and ammonium acetate 150 mmol L^{-1} and 250 mmol L^{-1} pH 7 respectively. The injection volume was 50 μL and the flow rate was 1 mL min^{-1}. The post-column solution was added at 0.15 mL min^{-1} using a Micro peripump MP-2 (Elemental Scientific, Inc, Omaha, USA). The LC was connected directly to the ICP-MS by using a PEEK tubing. A Thermo Element XR sector-field (SF) ICP-MS was used. The SF-ICP-MS was equipped with a Stable Sample Introduction (SSI) system and an Elemental Scientific ESI SC-E2 autosampler. The SF-ICP-MS tuning and resolution optimization was performed with a standard 1 ng mL^{-1} multi-element tuning solution. Integration of the chromatographic peaks was achieved using Origin (9.0). For post-column IDA quantification, a T-connector was used for connecting the outlet PEEK tubing from the LC with the ^{57}Fe spike solution, which was also connected to the nebulizer by PEEK tubing.

LC/MS/MS. A Thermo Dionex Ultimate 3000 coupled to a Thermo VELOS Pro mass spectrometer was used for the analysis of each sialoform in the serum. Peptides were loaded onto a Thermo Acclaim PepMap 100 C18, 3 μm, 100 Å loading column (75 μm × 2 cm) and then separated with a Thermo Acclaim PepMap 100 C18, 3 μm, 100 Å analytical column (75 μm × 15 cm) at a flow rate of 300 nL min^{-1}. Proteome Discover (version 1.4) was used for the identity confirmation of the different Tf sialoforms.

Procedures

Sample preparation. Samples were thawed to room temperature and then well mixed by gently rotating and inverting the ampoule before opening. For iron saturation of human serum Tf, six aliquots of SRM 909c (≈0.3 g and ≈0.1 g for post-column-IDA and species-specific IDA respectively) were diluted (1 : 1) with a solution containing 20 mmol L^{-1} ammonium bicarbonate, 150 mmol L^{-1} NaCl and 300 μm L^{-1} sodium citrate (pH 7.4). To this solution, 220 μL or 130 μL (for post-column-IDA and species-specific IDA respectively) of a 500 mmol L^{-1} sodium bicarbonate solution was added and then the sample was saturated with an appropriate amount of a freshly synthesized Fe–citrate solution (Fe–citrate was prepared at pH 7.4 using Fe standard solution (≈10 μg g^{-1}) and sodium citrate 0.9 mol L^{-1}) to a final concentration of 0.8 mmol L^{-1} of Fe, given the optimum Fe-to-citrate molar ratio 1 : 1000.[24] The mixture was stirred and incubated at 37 °C for 12 h (Fig. 1). Possible low molecular weight impurities and the excess Fe–citrate were removed by ultrafiltration through an Amicon centrifugal device (Amicon Ultra-2, Ultracel-10 kDa (Millipore)) and washed several times with Milli-Q water.

Sample analysis for quantification by post-column-IDA. The sample preparation for quantifying the Tf sialoforms by post-column-IDA consisted of taking the protein fraction (retained in the upper side of the filter after saturation of the Tf) and reconstituting with Milli-Q water to ≈400 μL. Approximately 50 μL of the sample was injected into the LC/SF-ISP-MS while a solution containing 150 ng g^{-1} of ^{57}Fe was continuously introduced through a T-piece (at 0.15 mL min^{-1}, using a peristaltic pump) at the end of the column and the mixture was nebulized into the plasma. Instrumental parameters can be seen in Table 1.

Table 1 Summary of the LC/ICP-MS chromatographic method

Dionex ICS-3000 Dual pump			
Column			
Dionex ProPac SAX-10 (4 mm × 250 mm)			
Mobile phases			
A	10 mmol L^{-1} tris-hydroxymethyl-aminomethane (Tris)		
B	50 mmol L^{-1} tris + 150 mmol L^{-1} ammonium acetate		
C	50 mmol L^{-1} tris + mmol L^{-1} ammonium acetate		
Gradient program			
Time (min)	%B	%C	Mobile phase (1.0 mL min^{-1})
0	0	0	
30	100	0	
35	0	100	
40	0	100	
41	0	0	
50	0	0	

Preparation of the isotopically enriched Fe-saturated Tf. For this purpose, the Apo-Tf was first dissolved with a solution containing 20 mmol L^{-1} ammonium bicarbonate, 150 mmol L^{-1} of

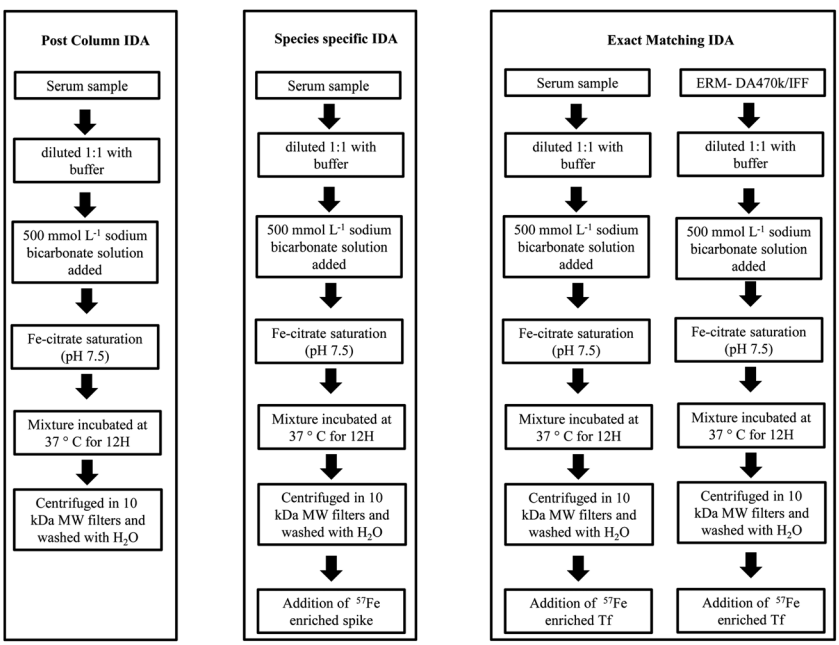

Fig. 1 Work flow illustrating the sample preparation process for the quantification of Tf sialoforms and total Tf.

NaCl and 300 μmol L^{-1} sodium citrate (pH 7.4) and then saturated with ^{57}Fe–citrate (following the same incubation procedure used for the natural Fe–citrate). In this case, the ^{57}Fe solution was first diluted with Tris 25 mmol L^{-1} and the pH was adjusted to 3 before adding the sodium citrate. Then, the pH was adjusted to 7.5 and the solution was allowed to equilibrate for 1 h at 25 °C before adding to the Apo-Tf.

Spike calibration. Two aliquots of ERM-DA470/IFCC (after being saturated with ^{56}Fe) were mixed with an aliquot of the isotopically enriched Tf spike. Duplicate injections of the two samples (diluted 1 : 2 with buffer A) were separated and detected by LC/SF-ICP-MS (at medium resolution). The working spike solution of ^{57}Fe–Tf was calibrated by reverse isotope dilution analysis using the ERM standard having a certified concentration of total Tf (2.36 ± 0.08 mg mL^{-1}). Considering the known stoichiometry of the iron saturated complex Fe_2–Tf (two mol of iron per mol of protein), the total concentration of iron can also be calculated from the certified Tf concentration. To determine the percentage of each isoform in the ^{57}Fe–Tf spike, an aliquot of the spike diluted 1 : 3 with buffer A was analyzed in triplicate by LC/SF-ICP-MS. Therefore, the concentration of iron on each sialoforms was obtained after applying these percentages to the total iron concentration of ^{57}Fe–Tf spike.

Sample analysis for quantification by species-specific IDA. Samples were prepared as described for post-column IDA (Fig. 1). After filtering the sample using the Amicon centrifugal devices, an aliquot of the synthesized spike (^{57}Fe–Tf), ≈100 μL, was added to each filter containing the samples. The mixture was filtered and washed with 500 μL of Milli-Q water before collecting ≈150 μL. Finally, 50 μL of the sample was injected into the LC/SF-ISP-MS.

Sample analysis for quantification by exact matching IDA. The calibration solution was obtained after saturating an aliquot of ERM-DA470/IFCC with Fe standard (SRM 3126a) following the same protocol applied for species-specific IDA (Fig. 1). Additionally, six aliquots of human serum (SRM 909c), ≈0.1 g, were saturated and mixed with the natural iron citrate solution following the same protocol described above. The individual sample was then spiked with an aliquot of ^{57}Fe-enriched Tf resulting in the calibration mixture (ERM-DA470/IFCC) and sample mixtures (SRM 909c). Additional samples ($n = 3$) of ERM-DA470/IFCC were prepared as quality control materials and run as unknown sample mixtures.

Verification of Tf sialoforms by ESI-MS after tryptic digestion. Fractions of each sialoform were collected from a saturated 909c sample using the separation mentioned previously. Each collected fraction was pre-concentrated and de-salted (using 10 kDa filters). For the identification of the proteins in each separated Tf-sialoform fraction by ESI-MS/MS, an aliquot of each isoform was first diluted with 20 μL of buffer (8 mol L^{-1} urea and 0.4 mol L^{-1} ammonium bicarbonate), then 5 μL of dithiothreitol (DTT) 45 mmol L^{-1} was added and incubated at 50 °C for 20 min to reduce the protein. The solution was allowed to cool to room temperature (RT) and 2 μL of 375 mmol L^{-1} iodoacetamide (IAA) was added and the solution was left in the dark at RT for 20 min. To reduce the amount of urea and ammonium bicarbonate Milli-Q water was added (35 μL). Next the digestion was carried out by adding trypsin (the ratio of trypsin : protein will be 1 : 50) and the solution was incubated overnight at 37 °C. To stop the digestion, 1 μL of trifluoroacetic acid (TFA) was added to each vial. The samples containing the digested protein of each sialoform were then desalted and pre-concentrated using C18 spin-columns (Pierce, Thermo Scientific).

Results and discussion

Separation of the Tf sialoforms

The separation of the Tf sialoforms was achieved after saturating the samples with Fe (SRM 3126a iron standard solution) to reduce the number of Tf isoforms occurring in the native serum sample. Fig. 2A shows the chromatogram of separated sialoforms obtained by LC/SF-ICP-MS (medium resolution) after injecting a serum sample completely saturated with Fe. Fig. 2B shows the chromatogram obtained by analyzing a saturated serum sample using the chromatographic method described by Del Castillo Busto *et al.*,[16] observing that this chromatographic method does not result in the base line resolved separation of the individual sialoforms. Therefore, comparing the separation achieved with this new chromatographic method with previously published chromatographic separations methods,[7,16] a significant improvement in the separation efficiency was observed, which can be translated to higher accuracy and greater precision on quantitative results.

The IFCC's WG-CDT has proposed disialo-Tf as the primary target analyte for CDT standardization.[8] Asialo-Tf would be an alternative target analyte because of its superior diagnostic efficiency compared to the sum of asialo-Tf, monosialo-Tf and disialo-Tf or even asialo-Tf + disialo-Tf for distinguishing not only between teetotalers and alcoholics, but also between moderate drinkers and alcoholic abusers.[25,26] The problem with current analytical methods for CDT analysis is that asialo-Tf is not measurable in abstinent and mild social drinking subjects, and is usually not detected even in relatively heavy alcohol consumers. Therefore, asialo-TF has not been considered a reliable marker of chronic alcohol abuse. However, as can be seen in Fig. 2A the higher separation efficiency and increased sensitivity demonstrate that asialo-Tf, monosialo-Tf and disialo-Tf are all detectable in the SRM 909c which makes the ICP-MS based method a superior alternative for diagnostic measurements of CDT. As the primary target molecule (disialo-Tf) in the WG-CDT reference method, the resolution between disialo-Tf and trisialo-Tf is of the most importance due especially in cases of samples containing high trisialo-Tf levels and common genetic Tf variants which cause the peaks of disialo-Tf and trisialo-Tf to be unresolved.[8] Samples in which the disialo-Tf and trisialo-Tf are unable to be resolved can lead to an underestimation of the peak area due to the calculations and therefore lead to poor clinical determinations of % CDT.

Post-column IDA analysis

Post-column IDA allows a calibrated flow of an isotopically enriched spike to be introduced post column and mixed with the separated sample prior to the ICP-MS. This method is of

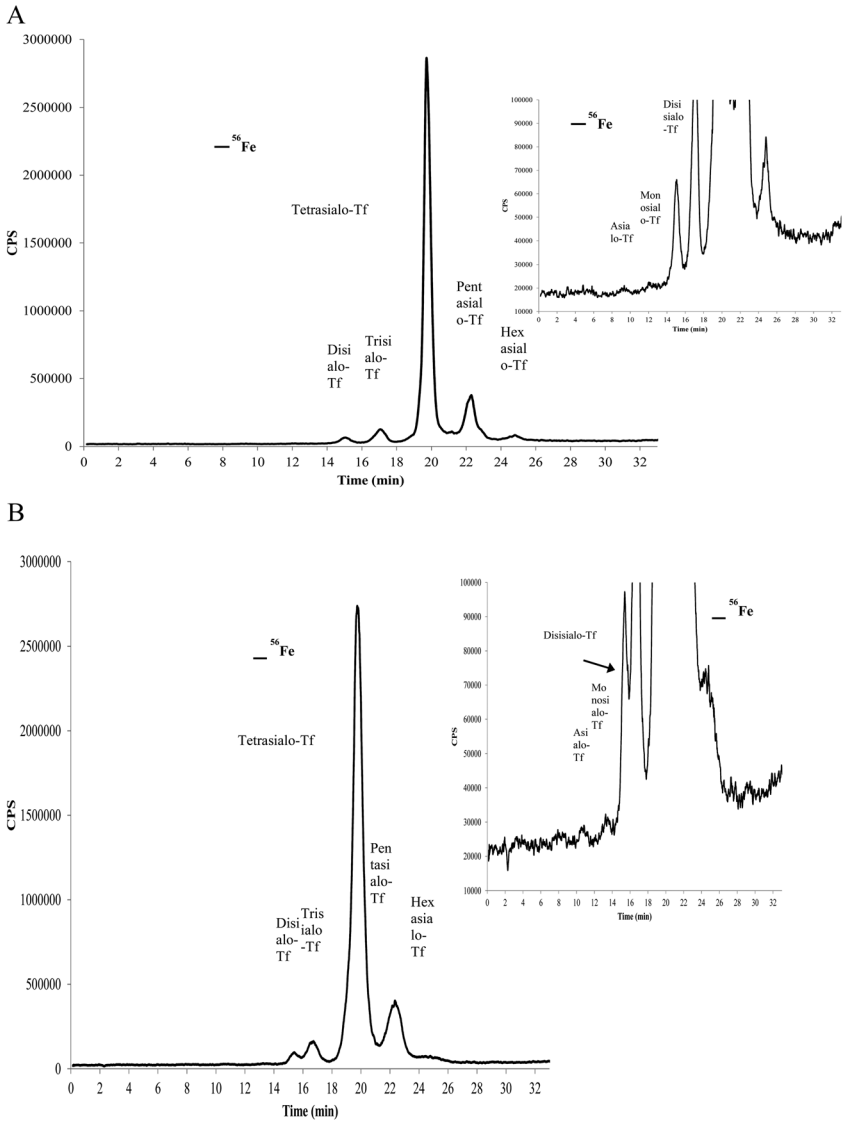

Fig. 2 (A) Example of Tf chromatographic separation of a Fe-saturated serum sample of SRM 909c human serum. (B) Example of Tf chromatographic separation of a Fe-saturated serum sample of SRM 909c human serum by a previously published method.[16]

great use in quantification of unknown species or when an isotopically enriched spike of a specific analyte is not available or difficult to produce. The main limitation of post-column IDA is that only the analyte eluting from the column is quantified and analyte losses during sample preparation are not accounted for. The intensity chromatogram (ion counts s^{-1}) was converted, after applying the post-column isotope dilution equation,[23] into a mass flow chromatogram (ng min^{-1}). The amount of Fe in each Tf sialoform was obtained by integrating the area under each peak using Origin 9.0. Using this approach, 6 aliquots from 6 different jars of SRM 909c were analyzed and the results are summarized in Table 2. As can be observed the precision of the method is <6%. In addition, the results for the ERM-DA470/IFCC analyzed as a quality control material are in good agreement with the certified value (Table 3). Considering the known stoichiometry of the iron saturated complex Fe_2–Tf (two mol of iron per mol of protein), the concentration of each sialoform (as protein) can also be calculated as well as the total concentration of Tf (sum of all the sialoforms) which can be seen in Table 2. In addition, the percentage of each sialoform in the serum (Table 4) can also be calculated and can be used as a SI traceable calibration material for the clinical measurement of % CDT (percentage of disialo-Tf) as defined by the WG-CDT.[8]

Species-specific IDA

Species-specific IDA allows for the isotopically enriched spike to be added to the sample prior to sample preparation and any loss can be accounted for both the analyte and spike because the analyte/spike ratio is fixed from the time both the analyte and spike are "equilibrated". Therefore, quantification of an analyte utilizing the species-specific mode is able to be directly linked to the original sample because both the sample and spike behave the same during sample preparation and analysis. The use of the

Table 2 Summary of results for individual Tf sialoforms and total Tf in SRM 909c human serum ($n = 6$)[a]

SRM 909c	Asialo-Tf	Disialo-Tf	Trisialo-Tf	Tetrasialo-Tf	Pentasialo-Tf	Hexasialo-Tf	Total Tf
ng g^{-1} as Fe							
Post-column IDA	2.5 ± 0.1	49.6 ± 2.0	120.7 ± 6.3	2310 ± 120	569.7 ± 35.3	53.9 ± 2.2	3106 ± 162
Species-specific IDA	2.9 ± 0.6	51.3 ± 5.7	129.6 ± 8.1	2595 ± 118	443.0 ± 40.2	50.6 ± 8.4	3272 ± 138
mg mL^{-1} as protein							
Post-column IDA[b]	0.0018 ± 0.0005	0.036 ± 0.001	0.088 ± 0.005	1.69 ± 0.09	0.42 ± 0.03	0.039 ± 0.002	2.28 ± 0.12
Species-specific IDA[b]	0.0022 ± 0.0005	0.038 ± 0.004	0.095 ± 0.006	1.90 ± 0.09	0.32 ± 0.03	0.038 ± 0.006	2.39 ± 0.10
Exact matching IDA[b]		—	—	—	—	—	2.19 ± 0.10

[a] The uncertainties associated with the measured values are expressed as expanded uncertainties, U, at the approximately 95% level of confidence ($k = 2$). [b] Density corrected using 1.027 mg mL^{-1}.

Table 3 Measured results of total Tf in ERM-DA470k/IFF mg mL^{-1} as protein[a]

ERM-DA470k/IFF[b]	Post-column IDA	Species-specific IDA	Exact matching IDA
Determined value	2.30 ± 0.11	2.42 ± 0.15	2.32 ± 0.09
Certified value	2.36 ± 0.08		

[a] The uncertainties associated with the measured values are expressed as expanded uncertainties, U, at the approximately 95% level of confidence ($k = 2$). [b] Density corrected using 1.0221 mg mL^{-1}.

species-specific IDA mode of quantification is only limited to the availability or production of the enriched spike of the specific analyte. In order to utilize species-specific IDA, an isotopically labeled species must be available or can be synthesized.[27] Since this isotopically enriched protein is not commercially available, the synthesis of an isotopically enriched ^{57}Fe has been carried out in our laboratory (as described in the Experimental section). Consequently, six aliquots of SRM 909c and 4 aliquots of ERM-DA470/IFCC were analyzed after spiking the samples with a known amount of ^{57}Fe–Tf spike. For the species-specific isotope dilution measurements, ^{57}Fe/^{56}Fe ratios from the integrated peak areas of each sialoform were downloaded to a Microsoft Excel spreadsheet for calculation of the individual Tf sialoform concentrations. The summary of results for the mass fraction determinations of each sialoform (as Fe) are listed in Table 2 along with the concentration of each sialoform (as protein) and the total Tf concentration (sum of all the sialoforms). While both the post-column (2.27 ± 0.12 mg mL^{-1}) and species-specific (2.39 ± 0.12 mg mL^{-1}) values for total Tf are in relatively good agreement, the slightly elevated Tf concentration observed with this methodology is most likely due to the fact that possible losses of the sample in the filter and on the column can be corrected (main advantage of the species-specific IDA). The results of the ERM-DA470/IFCC quality control material are also in good agreement with the certified value (Table 3).

Exact matching IDA

Exact matching IDA is a quantification method which aims to match both the isotopic ratios and signal intensities for the sample blend and the calibration blend thereby reducing many of the biases involved in the measurement of the ratios. The same amount of isotopically enriched spike is added to both a sample and a calibration standard with the amount of analyte in the calibration standard matched to be the same in the sample. This technique is limited by the fact that a previous knowledge of the analyte in an "unknown" sample must be utilized in order for an approximate match between sample and calibration standard to occur. In this case, ERM-DA470/IFCC was used as the calibration standard and directly compared to SRM 909c serum using the exact-matching IDA equation.[28] The average measured total Tf concentration in 909c was 2.19 mg mL^{-1} ± 0.10 mg mL^{-1} (Table 2). It can be seen in Table 2 that the value obtained by exact matching IDA overlaps with the total concentration (sum of all the sialoforms) obtained by both species-specific IDA and post-column IDA. All of the ICP-MS IDA quantitative results are also in good agreement with a separate analysis of total Tf in SRM 909c by QconCAT (1.92 mg mL^{-1})

Table 4 Percentages of individual Tf sialoforms in SRM 909c human serum % sialoforms[a]

Sample	Asialo-Tf	Disialo-Tf	Trisialo-Tf	Tetrasialo-Tf	Pentasialo-Tf	Hexasialo-Tf
Low serum pool measured value ($n = 3$)	0.11 ± 0.02	1.6 ± 0.1	4.2 ± 0.1	77.0 ± 0.7	15.6 ± 0.3	2.0 ± 0.1
Low serum pool reference value	—	1.2 ± 0.3	3.5 ± 0.4	77.9 ± 1.1	16.4 ± 1.2	1.5 ± 0.6
High serum pool measured value ($n = 3$)	0.29 ± 0.04	3.8 ± 0.1	5.0 ± 0.1	73.6 ± 0.2	15.4 ± 0.1	1.9 ± 0.2
High serum pool reference value	0.3 ± 0.1	3.4 ± 0.5	4.2 ± 0.4	75.3 ± 1.7	15.9 ± 1.2	1.4 ± 0.6
SRM 909c post-column IDA ($n = 6$)	0.08 ± 0.02	1.6 ± 0.1	3.9 ± 0.1	74.4 ± 0.3	18.3 ± 0.4	1.7 ± 0.1
SRM 909c species-specific IDA ($n = 6$)	0.09 ± 0.02	1.6 ± 0.2	4.0 ± 0.3	79.3 ± 3.4	13.6 ± 1.3	1.6 ± 0.3

[a] The uncertainties associated with the measured values are expressed as expanded uncertainties, U, at the approximately 95% level of confidence ($k = 2$).

and synthetic internal standards (2.02 mg mL^{-1}) quantification.[29] The additional ERM-DA470/IFCC quantitative measurements are listed in Table 3.

Identification of each separated Tf sialoform by ESI-MS

Proteins of each separated Tf sialoform fraction were identified by a shotgun proteomic approach based on a "data dependent acquisition" (DDA) strategy. The DDA strategy applied consisted of detecting ions through a large 350–2000 *m*/*z* range and by selecting the 10 most abundant ions of the MS scan for fragmentation. Once MS/MS spectra had been acquired, a sequence-searching experiment was performed on the MS/MS spectra with Proteome Discover search engine and a human protein database including variable modifications such as oxidation of methionine and alkylation of cysteine residues. The coverage factor of Tf obtained for all the fractions was >79%, listed in the ESI.†

Application of % CDT in clinical measurements

The relative quantification results (mass fraction of individual transferrin sialoforms/mass fraction of total Tf × 100) of both clinical control materials (low serum pool and high serum pool) are shown in Table 4 along with the % conversions of SRM 909c. Despite the slightly elevated value of total Tf using species-specific IDA, the percentages of the sialoforms are in excellent agreement. It can be observed that there is an increase in the disialo-Tf in the high pool, as well as an increase in the asialo-Tf. This is consistent with other clinical measurements which demonstrate an increase in both the asialo-Tf and disialo-Tf sialoforms in positive % CDT patients of moderate to high alcohol consumption. The 1.6% disialo-Tf (% CDT) value for SRM 909c is consistent with the low serum pool and therefore may be of use as a control material for low alcohol consumers and/or social drinkers. Additionally, Table 4 shows the range of % of each sialoform obtained by the MUSC Institute of Psychiatry Clinical Neurobiology Laboratory using the WG-CDT LC-UV/vis reference method for analyzing CDT in serum[7] demonstrating that the ICP-MS measured values are in good agreement with the clinical reference values for both materials. The improved separation of Tf-sialofoms in combination with the higher sensitivity ICP-MS yields a significant improvement in the ability to detect the lower abundant Tf sialoforms as can be seen with the quantification of the asialo-Tf peak even in the low control group serum (Table 2).

Conclusions

This work demonstrates a significant improvement of the separation of the different Tf sialoforms compared to previously published methods, which can be translated to greater accuracy and increased precision on absolute and relative quantitative results. Additionally, the higher sensitivity of the ICP-MS also allows the detection and quantification (absolute and relative) of asialo-Tf which could be used as an alternative target analyte for % CDT clinical measurements. The quantitative results were obtained after applying different isotope dilution techniques including both post-column IDA and species-specific IDA to obtain the concentration of the different Tf sialoforms. Total Tf concentrations were also determined by all three IDA techniques. The total Tf values obtained after applying species-specific IDA are slightly higher than the ones obtained by post-column IDA which can be attributed to possible column losses with the former methodology. All the measurements are validated with the measurement of a Certified Reference Material (ERM-DA470-IFF) with a SI traceable certified concentration value of Tf. Furthermore, the established method points to the possibility of providing a commercially available and well-characterized Tf standard in human serum (SRM-909c) to which clinical diagnostic measurements of total Tf and each individual sialoform (% CDT) can be traceable.

Disclaimer

Certain commercial items are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the equipment identified is necessarily the best for the purpose.

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