

Rapid Determination of Transferrin Isoforms by Immunoaffinity Liquid Chromatography and Electrospray Mass Spectrometry

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Background: Congenital disorders of glycosylation (CDG) are autosomal recessive disorders that produce increased serum carbohydrate-deficient transferrin (CDT) isoforms. Methods to resolve CDT from fully glycosylated transferrin (Trf) have been based on a neutral shift in the isoelectric focusing (IEF) pattern or on a reduction in the negative charge, allowing resolution by anion-exchange chromatography. Our purpose was to develop a method of resolution and relative quantification of Trf isoforms using online immunoaffinity liquid chromatography–mass spectrometry (LC-MS).

Methods: Serum (25 μ L) was diluted with 100 μ L of water before application to an immunoaffinity column that sequestered Trf isoforms. Trf isoforms were eluted from the immunoaffinity column, concentrated on a C₄ column, eluted from the C₄ column, and introduced into the mass spectrometer. Analysis of the Trf isoforms was entirely automated and completed in <10 min per sample.

Results: The LC-MS method demonstrated that the major abnormal Trf isoforms in CDG lack one complete oligosaccharide structure (mono-oligosaccharide) or both oligosaccharide structures (a-oligosaccharide), but not the sialic acids, as presumed on the basis of IEF methods. Calculation of relative ratios among three possible species (mono-/di-oligosaccharide and a-/di-oligosaccharide) is reproducible [mean intra- and inter-assay CVs were 9.3% (n = 10) and 10% (n = 5), respectively]. A reference range for patients <18 years was determined by analysis of 209 samples (for mono-/di-

oligosaccharide, the median was 0.041 and the range was 0.018–0.083; for a-/di-oligosaccharide, the median was 0.007 and the range was 0.002–0.036). Comparison of data obtained with an affinity chromatography-IEF method and the LC-MS method demonstrated equivalence in the interpreted results (n = 170).

Conclusions: Advantages of the LC-MS method include improved sensitivity, minimal sample preparation, and an analysis time of <10 min. The method was automated, which allowed high throughput, with >100 samples analyzed in a single day. Moreover, the nature of the oligosaccharide defect in CDG is accurately reflected by mass resolution, and subtle oligosaccharide truncations may also be detected by this method.

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Transferrin (Trf)⁴ is a serum glycoprotein that is synthesized in the liver and consists of a single polypeptide chain of 679 amino acids (1). It is an important iron transport protein that possesses two iron-binding sites (Fig. 1) (2). Abnormal Trf isoforms, commonly referred to as carbohydrate-deficient Trfs (CDTs), are biochemical markers for congenital disorders of glycosylation (CDG) and chronic alcohol consumption (3, 4). CDG are autosomal recessive disorders presenting in infancy or early childhood with neurodevelopmental delay (5). Dysmorphic features such as unusual distributions of subcutaneous fat and cerebellar hypoplasia also occur in children affected with the most common clinical phenotype. Trf is the preferred biochemical marker for diagnostic analysis, primarily because of its abundance in serum. However,

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⁴ Nonstandard abbreviations: Trf, transferrin; CDT, carbohydrate-deficient transferrin; CDG, congenital disorders of glycosylation; PMM, phosphomannomutase; PMI, phosphomannose isomerase; IEF, isoelectric focusing; LC-MS, liquid chromatography–mass spectrometry; and PBS, phosphate-buffered saline.

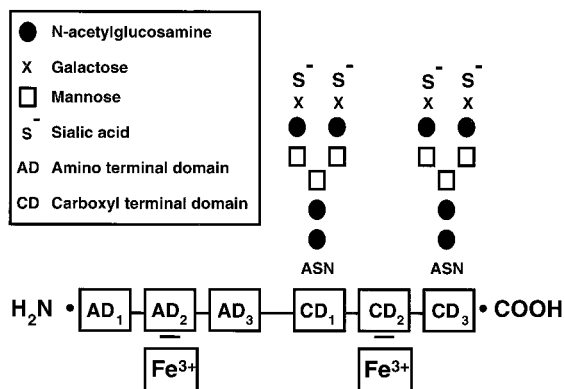


Fig. 1. Domain structure of Trf.

The primary structure contains three NH_2 -terminal domains (AD_1 – AD_3) and three COOH -terminal domains (CD_1 – CD_3). Iron-binding sites and asparagine glycosylation sites are depicted.

other glycosylated proteins, including peptide hormones, are abnormal in CDG as well and are the likely cause of the endocrine function abnormalities that are among the constellation of symptoms (6). The biochemical deficiencies that cause the most frequent forms of CDG have been identified and are caused by mutations in the phosphomannomutase (PMM) and phosphomannose isomerase (PMI) genes (7, 8).

Isoelectric focusing (IEF) is the most commonly used method for detection of CDT and is considered the gold standard for the diagnosis of CDG (9). Typically, five sialic acid residues contribute to the acidic pI of Trf, and loss of these residues causes a basic shift within the pI of 5.2–5.9. Current methods for specific identification of Trf isoforms use combinations of IEF in conjunction with Western blotting or immunopurification-IEF followed by Coomassie staining (4). Demonstration of CDT for CDG diagnosis by either of the above sequences is adequately done by IEF. More recently, microcolumn separation followed by a turbidimetric immunoassay or HPLC has been introduced to report CDT as a relative amount compared with total serum Trf (10). Ion exchange in a HPLC configuration has also been demonstrated (11). As with IEF, resolution of Trf isoforms by ion-exchange chromatography is based on charge and is not affected by neutral monosaccharide moieties. This has led to the conclusion that CDT is a Trf lacking sialic acid residues. This structure is not consistent with the molecular weight measurements we attribute to CDT in CDG using mass spectrometry.

Given the increasing clinical interest in CDG and the gradual transition of this procedure to a high-volume test, replacing the affinity chromatography-IEF method is highly desirable in reducing turnaround time. We have, therefore, developed a fully automated online immunoaffinity liquid chromatography–mass spectrometry (LC-MS) method for routine detection of Trf isoforms.

Materials and Methods

MATERIALS

POROS™-aldehyde self pack medium, POROS-aldehyde loading buffer, POROS-aldehyde high-sulfate buffer, sodium cyanoborohydride, and POROS-aldehyde capping buffer were purchased from PerSeptive Biosystems. Rabbit anti-human Trf used in the immunoaffinity column was purchased from Dako. The C.128 precolumn used for immunoaffinity was purchased from Upchurch. Slide-A-Lyzer dialysis cassettes (10 000 molecular weight cutoff) and syringe and needle accessories were purchased from Pierce. Apo-Trf was purchased from Sigma. All other chemicals and solvents were of the highest analytical grade available from commercial sources and were used without further purification.

IMMUNOAFFINITY COLUMN PREPARATION

Buffers and solutions from PerSeptive Biosystems were prepared according to directions provided by the manufacturer. Rabbit anti-human Trf (14 mg) was dialyzed overnight against 1 L of phosphate-buffered saline (PBS; 0.01 mol/L phosphate-0.150 mol/L NaCl, pH 7.4) in a Slide-A-Lyzer dialysis cassette. After overnight dialysis, rabbit anti-human Trf was added to 4 mL of PBS, slowly mixed with 2.5 mL of POROS-aldehyde high-sulfate buffer, and then mixed with sodium cyanoborohydride to a final concentration of 5 g/L. POROS-aldehyde self pack medium was added (150 mg), and the entire solution was rocked gently for 1 min. POROS-aldehyde high-sulfate buffer was added (1 mL), and the solution was agitated for another 5 min. Additional 1-mL increments of POROS-aldehyde high-sulfate buffer were added every 5 min for a total of 5 mL. The solution was rocked for 90 min at room temperature. The immobilized ligand was filtered using a 10–20 μm sintered glass funnel, resuspended in 10 mL of POROS-aldehyde capping buffer, and rocked for 30 min at room temperature. The immobilized ligand was then washed using 50 mL of POROS-aldehyde loading buffer, 50 mL of 1 mol/L NaCl, and 50 mL of POROS-aldehyde loading buffer before being stored in 1 mL of POROS-aldehyde loading buffer. Five C.128 columns (20 \times 1 mm) were packed at a flow rate of 2 mL/min with the immobilized ligand (17 μL /column) using POROS-aldehyde loading buffer. Columns stored for later use were washed with 0.2 g/L sodium azide in loading buffer.

SAMPLE PREPARATION

A 25- μL serum sample was mixed with 100 μL of reverse-osmosis H_2O (1:5 dilution by volume). After vortex-mixing, a 100- μL portion of the dilution was transferred to an autosampler vial for analysis.

METHODS

LC-MS method. Method development and validation were performed using an API 3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex) equipped with the TurboIon-Spray ionization probe source (operated at 5500 V). Periph-

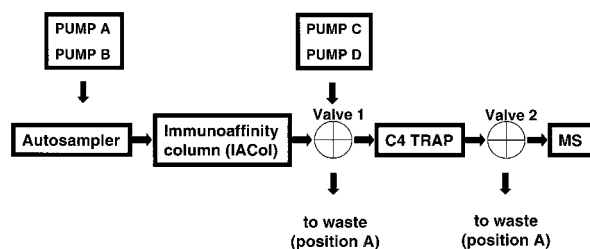


Fig. 2. LC-MS configuration, including pumps, autosampler, immunoaffinity column, C_4 column, and switching valves.

Pump A, binding buffer (PBS); *pump B*, elution buffer (0.1 mol/L glycine and 20 mL/L acetic acid, pH 2.5); *pump C*, 10 mL/L acetic acid-methanol-acetonitrile (98:1:1 by volume); *pump D*, 0.5 mL/L acetic acid in 0.2 trifluoroacetic acid-methanol-acetonitrile (5:48:48 by volume). *Pumps A and B*, Shimadzu LC-10ADvp; *pumps C and D*, PE Sciex Series 200 Micropump. *Valve 1*, column switching valve; *valve 2*, diverting valve.

erals included two Perkin-Elmer Series 200 Micropumps, a Perkin-Elmer Series 200 Autosampler, and a Shimadzu System Controller (SCL-10Avp), which controlled two Shimadzu Liquid Chromatography LC-10ADvp pumps and two Valco two-position actuator control modules.

A 5- μ L aliquot of the diluted serum was applied to the immunoaffinity column (20 \times 1 mm column containing anti-human Trf coupled to POROS-aldehyde medium) in PBS (pH 7.4) at a flow rate of 100 μ L/min for 2 min. All other serum components were diverted to waste because of the configuration of valve 1 (position A). At 2 min into the run, valve 1 was switched to position B, and Trf

isoforms were subsequently eluted from the immunoaffinity column for 2 min with 100 mmol/L glycine containing 20 mL/L acetic acid buffer (pH 2.5; flow rate, 100 μ L/min) and concentrated on a C_4 cartridge [Widapore C_4 (butyl); 4 \times 2.0 mm (i.d.); Phenomenex]. The C_4 column was then washed with 10 mL/L acetic acid-methanol-acetonitrile (98:1:1 by volume) at a flow rate of 100 μ L/min for 2 min to remove excess phosphate and other buffer components that might suppress MS response. Valve 2 was switched to position B 6 min into the run, and Trf isoforms were eluted from the C_4 column and introduced into the TurboIonSpray source using 5 mL/L acetic acid in 0.2 g/L trifluoroacetic acid-methanol-acetonitrile (5:48:48 by volume) at a flow rate of 100 μ L/min (Fig. 2). The TurboIonSpray source was operated with turbo gas on (6 L/min; sensor temperature, 150 $^{\circ}$ C) with the effluent flow splitting at 1:2. The mass spectrometer was operated in Q1 scan mode (m/z 2000 to m/z 3000) with a Trf retention time of \sim 7 min and total instrument acquisition time of 9.5 min/sample. After completion of development, the method was also validated on an API 150 single quadrupole LC-MS system (Perkin-Elmer Sciex) equipped with the same TurboIonSpray ionization probe source and peripherals.

Data were acquired and processed using MassChrom software (Ver. 1.1.2; Perkin-Elmer Sciex), including Bio-MultiView (Ver. 1.3.1; Perkin-Elmer Sciex). The Bio-

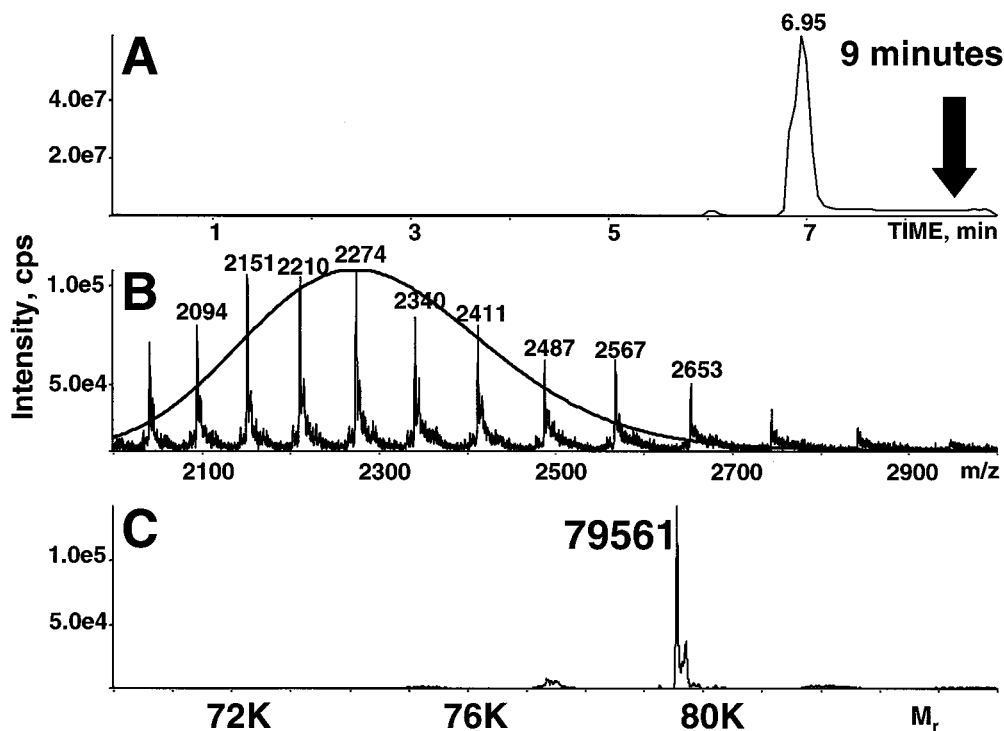


Fig. 3. LC-MS analysis of control serum.

(A), total-ion chromatogram with Trf eluting at 7 min in a 9-min analysis. (B), multiply charged ion distribution of Trf. (C), BioSpec-Reconstruct deconvolutes charge distribution raw data to reconstructed mass data. A single peak at 79 561 Da is seen in control serum, which represents the intact dioligosaccharide tetraantennary glycoprotein.

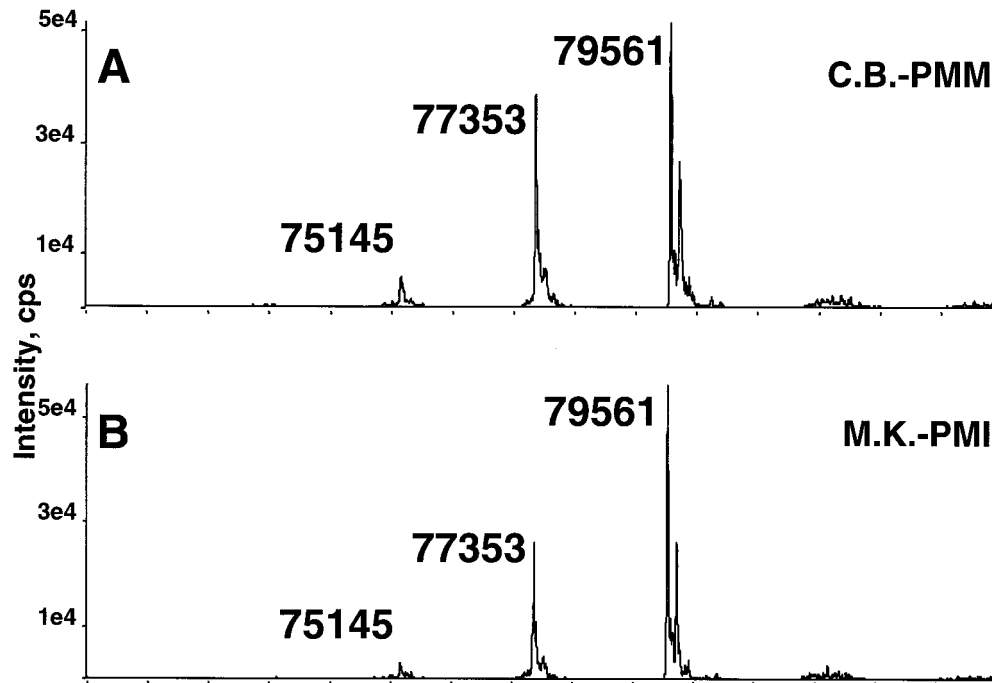


Fig. 4. LC-MS analysis of CDG serum.

Three distinct peaks are seen in CDGS serum at 79 561, 77 353, and 75 145 Da, which represent the intact glycoprotein, loss of one oligosaccharide (loss of ~2208 Da), and loss of two oligosaccharides (loss of ~4416 Da), respectively. Chromatograms shown are from patients affected by PMM (A) and PMI (B) deficiency as determined by enzyme analysis.

SpecTM-Reconstruct algorithm was used to deconvolute the charge distribution raw data to reconstructed mass data. Specifically, multiply charged spectra were transformed through five iterations, using an input data range of m/z 2000–3000 and an output data range of 74 000 to 81 000 Da. Relative quantification of CDT was achieved by comparing a-oligosaccharide Trf and mono-oligosaccharide Trf with di-oligosaccharide Trf.

IEF method. For method comparison, affinity chromatography-IEF was adapted from previously published procedures (4). Rabbit anti-human Trf coupled to Sepharose was prepared by mixing rabbit anti-human Trf with CNBr-activated Sepharose 4B (Pharmacia Amersham Biotech) under standard coupling conditions (12). The anti-human Trf was stored suspended in 0.1 mol/L citrate-0.025 mol/L phosphate, pH 7.2 (1:1 by volume).

Trf was isolated from 1 mL of serum by mixing it with 2 mL of antibody-Sepharose suspension for 2 h at room

temperature. After binding, the resin and liquid were transferred to a 4-mL syringe column fitted with a frit (Alltech). The binding mixture was expelled, and unbound proteins were eluted with eight 2-mL washes of the binding buffer (0.01 mol/L citrate-0.025 mol/L phosphate, pH 7.2). The Trf was eluted with four 1-mL washes of elution buffer (0.1 mol/L citrate-0.025 mol/L phosphate, pH 2.9), and immediately neutralized with Na_2HPO_4 to a pH of 7.2. After overnight dialysis of the neutralized eluate vs 4 L of 0.5 mol/L Na_2HPO_4 , the Trf was iron saturated by incubation with 50 mL of 20 mmol/L ferric citrate for 1 h at 37 °C. The Trf was concentrated in Amicon Centricon YM-30 (Millipore) microconcentrators, and protein was determined by the Lowry method (13). Serum Trf preparations were diluted to 0.5–1.0 g/L protein depending on their concentration, and 15 μL was applied to the focusing gels. The IEF gels consisted of 6% acrylamide, pH 3.0–10.0. The gels were focused until 1940 V-h were reached at 5 W (~2 h). The gels were fixed with a mixture of 115 g/L trichloroacetic acid and

Table 1. Stability of reconstructed masses.

	Within run ^a		Between run ^b	
	Median	Range	Median	Range
A-oligo	75 150	75 141–75 157	75 152	75 145–75 157
Mono-oligo	77 355	77 347–77 364	77 358	77 356–77 361
Di-oligo	79 561	79 553–79 565	79 562	79 557–79 568

^a Represents reconstructed masses from typical analytic run of 10 different specimens.

^b Represents reconstructed masses for patient specimens selected at random from 10 different analytic runs.

Table 2. Precision of LC-MS method.

Sample ^a	Ratio	Initial value	CV, %	
			Intraassay, n = 10	Interassay, n = 5
Normal	Mono-/di-	0.059	8.0	8.3
Increased	Mono-/di-	0.391	9.7	3.6
Increased	Mono-/di-	1.100	8.6	12
Increased	A-/di-	0.203	11	18

^a Samples selected from serum samples submitted for CDT determination.

35 g/L 5-sulfosalicylic acid for 30 min, rinsed with reverse-osmosis H₂O, and then soaked in reverse-osmosis H₂O for 1 h. The Trf bands were visualized by staining with 5 g/L Coomassie Brilliant Blue in 250 mL/L ethanol and 100 mL/L acetic acid and destaining for 1 h with 400 mL/L methanol and 100 mL/L acetic acid. The gels were preserved for photography and densitometry by soaking in 25 mL/L glycerol for 15 min. Scanning was accomplished using the Helena Rep densitometer (Helena Laboratories). Results were considered abnormal if the asialo and mono-asialo bands were >5% and >6%, respectively, and/or the disialo band was >15% of the total Trf as determined by the densitometer (4). The total time to setup and process a batch of 24 samples was 3 days, including preparation of acrylamide gels 24–48 h before use.

Results

The total-ion chromatogram obtained after a 5- μ L injection of 1:5 diluted serum is shown in Fig. 3A, with Trf eluted at ~7 min in a 9-min analysis. The BioSpec-Reconstruct algorithm deconvolutes charge distribution raw data (Fig. 3B) to reconstructed mass data with normal control serum showing a single ion at 79 561 Da (Fig. 3C), a value in close agreement to the calculated mass of di-oligosaccharide Trf (79 555.415 Da) as reported in the Swiss Protein Data Base. Trf immunopurified from serum of patients diagnosed with CDG (both PMM and PMI) revealed three distinct species when analyzed by LC-MS (Fig. 4). The three ions at 79 561, 77 353, and 75 145 Da present in CDG serum correspond to the intact glycoprotein, loss of one oligosaccharide moiety (loss of ~2208 Da), and loss of two oligosaccharide moieties (loss of ~4416 Da), respectively. These isoforms correspond to those called disialo and asialo bands, respectively, when

resolved by IEF. The within- and between-run stability of the reconstructed masses are summarized in Table 1.

PRECISION AND STABILITY

Precision data were determined by replicate analyses of one normal and three abnormal serum specimens on a single day (n = 10), followed by a single analysis on subsequent days (n = 5), and are summarized in Table 2 (mean intra- and interassay CVs were 9.3% and 10%, respectively). The data show that inter- and intraassay CVs were reproducible over control and abnormal oligosaccharide ratio ranges.

The stability of Trf isoforms after initial serum dilution was determined by repeat injections of four samples covering control and abnormal oligosaccharide ratio ranges. Samples were stored at room temperature between injections. Table 3 shows data obtained and indicates stability of Trf isoforms up to 96 h after initial dilution.

REFERENCE RANGE DETERMINATION

The reference range for pediatric patients (median age, 3 years; range, 1 day to 17 years) was determined using 209 control sera (for the mono-/di-oligosaccharide ratio, the median was 0.041 and the range was 0.018–0.083; for the a-/di-oligosaccharide ratio, the median was 0.007 and the range was 0.002–0.036). The distribution of results is shown in Fig. 5 and compared with the 24 CDG cases described below.

METHOD COMPARISON

Unused portions of 170 serum samples routinely analyzed by affinity chromatography-IEF were reanalyzed by LC-MS. Of these samples, 24 were from patients for whom either the enzyme deficiency (PMM or PMI) was detected (n = 12) or the original abnormal IEF pattern was confirmed by an independent laboratory (n = 12). Result interpretation based on the isoform ratio data indicated equivalence for both control and CDG specimens vs the IEF method (data not shown).

Discussion

The prevalent cause of CDG is an inherited deficiency of the enzyme PMM. This enzyme is required to synthesize mannose-1-phosphate, which is a precursor to GDP-man-

Table 3. Stability of Trf isoforms following sample dilution.

Serum samples ^b	Trf isoforms, relative ratio amount ^a				
	0 h	48 h	72 h	96 h	Mean \pm SD
1-Mono-/di-	0.059	0.053	0.048	0.053	0.053 \pm 0.005
2-Mono-/di-	0.391	0.418	0.458	0.446	0.428 \pm 0.030
3-Mono-/di-	1.100	0.954	0.985	0.859	0.975 \pm 0.099
4-A-/di-	0.203	0.216	0.242	0.239	0.225 \pm 0.019

^a Samples were analyzed immediately after dilution (time 0 h) and after the times indicated above. Between analyses, they were stored at room temperature in the autosampler vials.

^b Samples selected from serum specimens submitted for CDT determination.

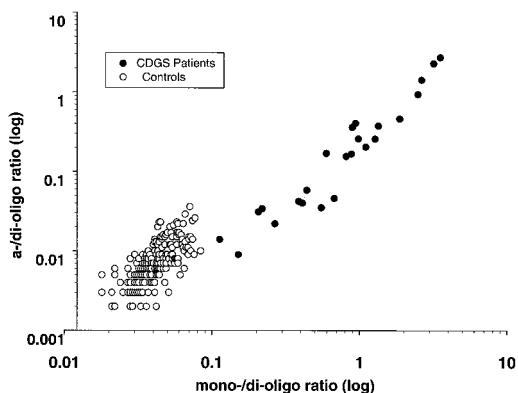


Fig. 5. Method comparison of samples analyzed by affinity chromatography-IEF and LC-MS.

Pediatric specimens used to establish reference ranges were waste serum from patients referred to our laboratory who had no apparent metabolic and nutritional abnormalities ($n = 209$). Twenty-four serum samples that tested positive for CDG by affinity chromatography-IEF method were also positive by the LC-MS method.

nose, the form of mannose used in glycosylation of the dolichol oligosaccharide required for N-glycosylation of proteins (6). Our results are supportive of the conclusion that Trf in CDG patients is completely missing one or both oligosaccharides (14–16). This suggests that the transfer of oligosaccharides to asparagine is depressed if sufficient mature oligosaccharide is not available. We propose that failure to produce adequate mannose-1-phosphate leads to functional deficiency of mannosylated oligosaccharide and that CDG patients with PMM or PMI deficiency have Trf with identical structural defects. The reproducible observation of masses of 77 353 Da (mono-oligosaccharide) and 75 145 Da (a-oligosaccharide) substantiate the abnormal structure of Trf. It is clear from our data that incomplete transfer of sialic acid to the ends of the oligosaccharides or remodeling of oligosaccharide structures, which would occur in the Golgi apparatus, is not the likely cause for the appearance of CDT in CDG.

In conclusion, LC-MS allows the detection of Trf isoforms in as little as 5 μ L of serum with an analysis time <10 min/sample, two major improvements over IEF (required volume, 1 mL) or turbidimetric immunoassay (100 μ L), which also paves the way to a possible application of this method to the analysis of dried blood spots. This method does not require iron loading, which is an important advantage because it is well documented that the loss of one iron residue from Trf causes an artifactual pI shift nearly equivalent to the loss of a sialic acid residue (9), mimicking a CDG-like pattern. LC-MS offers numerous technical advantages over our current affinity chromatography-IEF method and other methods. It is rapid, requires virtually no sample preparation, and the online steps have been completely automated. The use of acrylamide and IEF for routine clinical tests is undesirable for both safety and convenience reasons. Although the affinity chromatography-IEF method used previously in our laboratory

requires 3–4 days for completion, much of the time is not “hands-on”, and shortening of the process could be achieved; however, it never approaches an output of >100 samples per day. In our estimates, personnel effort and total cost (supplies and equipment depreciation cost, based on the less expensive single-stage LC-MS system) were reduced by 75% and 93%, respectively.

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