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Improved HPLC Method for Carbohydrate-deficient Transferrin in Serum

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Background: **There is need for a reference method for transferrin glycoforms in serum to which routine immunologic methods for the alcohol marker carbohydrate-deficient transferrin (CDT) can be traceable. We describe an improved HPLC method for transferrin glycoforms.**

Methods: **Transferrin was iron-saturated by mixing the serum with ferric nitrilotriacetic acid, and lipoproteins were precipitated with dextran sulfate and calcium chloride. Separation of glycoforms was performed on a SOURCE 15Q anion-exchange column using salt gradient elution. Quantification relied on selective absorbance of the iron–transferrin complex at 470 nm. The relative amount of each glycoform was calculated as a percentage of the area under the curve, using baseline integration.**

Results: **The HPLC system provided reproducible separation and quantification of the asialo-, monosialo-, disialo-, trisialo-, tetrasialo-, pentasialo-, and hexasialotransferrin glycoforms. Most importantly, disialo- and trisialotransferrin were almost baseline separated. The intra- and interassay CV for disialotransferrin were <5%. Serum and the pretreated samples were stable for at least 2 days at 22 or 4 °C. Sera from 132 healthy controls contained [mean (SD)] 1.16 (0.25)% disialotransferrin, 4.77 (1.36)% trisialotransferrin, 80.18 (2.01)% tetrasialotransferrin, and 13.88 (1.69)% pentasialo- + hexasialotransferrin. In some cases of a high (>6%) trisialotransferrin, monosialotransferrin was detected at <0.25%. Asialotransferrin was not detected in control sera, but was detected in 57% of chronic heavy drinkers and in 62% of sera with ≥2% disialotransferrin.**

Conclusions: **The HPLC method fulfills the requirements of a preliminary reference method for CDT and should work for any combination of serum transferrin glycoforms. This method could also be useful for confirming positive CDT results by immunoassays in medico-legal cases.**

Carbohydrate-deficient transferrin (CDT)[4] is a biochemical marker used for identifying recent, regular high alcohol consumption and for monitoring abstinence during outpatient treatment. CDT refers to an altered glycoform, or isoform, profile of the iron-transport glycoprotein transferrin in serum and occurs as a result of alcohol-induced changes in the carbohydrate composition of the molecule *(1)*. Transferrin glycoforms traditionally have been named depending on the number of terminal sialic acid residues on the N-linked oligosaccharide chains (glycans), which may be biantennary, triantennary, or even tetraantennary in structure *(2)*. The major transferrin glycoform under normal conditions contains two disialylated biantennary glycans (i.e., a total of four terminal sialic acid residues) and was accordingly named tetrasialotransferrin.

Individuals who have been drinking excessively over the past 2 or more weeks typically show increased relative amounts of disialotransferrin and, in cases of a high disialotransferrin, of asialotransferrin *(1, 3)*. These glycoforms were originally believed to contain truncated N-glycans deficient only in the terminal saccharide(s) *(1)*, but more recent studies have demonstrated that the major modification observed is a loss of one (disialotransferrin) or both (asialotransferrin) of the entire glycans and, consequently, of the terminal sialic acid residues *(4–7)*. When drinking is discontinued, the glycoform pattern of serum transferrin slowly normalizes with a half-life of

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[4] Nonstandard abbreviations: CDT, carbohydrate-deficient transferrin; IEF, isoelectric focusing; CE, capillary electrophoresis; AUDIT, Alcohol Use Disorders Identification Test; FeNTA, ferric nitrilotriacetic acid; AUC, area under the curve; CRP, C-reactive protein; and CI, confidence interval.

~1.5–2 weeks *(1, 8)*, and the time to reach a stable baseline value could require abstinence for 1 month or longer *(9)*.

The main asset of CDT compared with the conventional biochemical indicators of chronic alcohol abuse, such as the liver enzyme γ-glutamyltransferase and the mean corpuscular volume of erythrocytes, is its high specificity for alcohol abuse with a resulting low rate of false-positive identifications *(10, 11)*. However, drawbacks include the lack of standardization regarding the procedures applied for determination and in the definition of CDT. Transferrin glycoforms were originally separated, identified, and quantified by isoelectric focusing (IEF), and those glycoforms that were isoelectric at or above pH 5.7 after complete iron saturation, corresponding to disialo-, monosialo- and asialotransferrin, were collectively named CDT *(1)*. For routine determination of CDT, several commercial and noncommercial methods based on ion-exchange chromatographic separation on minicolumns followed by immunoassay *(12, 13)*, HPLC *(8, 14)*, and capillary electrophoresis (CE) *(15, 16)* are in use. Many of these tests differ in that they include heterogeneous mixtures of the asialo-, disialo-, monosialo-, and/or trisialotransferrin glycoforms in the "CDT" fraction *(3)*, and the test values are given in various absolute (e.g., mg or units of CDT per liter of serum) and relative (e.g., percentage of CDT to total transferrin or of disialotransferrin to tetrasialotransferrin) amounts. This inconsistency has caused much confusion over the years, related to both the discrepancies among methods and their ability to distinguish between a normal and an increased value (i.e., the sensitivity and specificity for high alcohol intake), and has often hampered direct comparison of results between studies *(17)*.

The need for international standardization of CDT determinations was highlighted during a meeting in Berlin, Germany, in 2000, which was attended by clinical chemists and alcohol researchers from several countries *(3)*, and this need has recently been re-addressed *(18)*. The participants of the standardization meeting agreed to develop a reference method for CDT based on a HPLC technique to which any routine method can be traceable. HPLC was chosen because it allows for reproducible separation and visible detection of the different transferrin glycoforms and because genetic transferrin variants and glycoform types that may cause incorrect determination of CDT with the minicolumn immunoassays are readily identified from a unique peak pattern in the chromatograms *(3)*. Furthermore, because HPLC is common in many hospital and research laboratories and a suitable analytical column and all chemicals to be used are commercially available, transferability of the method was indicated to be high.

This study reports on the development and validation of an improved HPLC method for measurement of CDT in serum, based on anion-exchange chromatographic separation of the different transferrin glycoforms followed by photometric detection.

Materials and Methods

SERUM SAMPLES

The serum samples used for technical evaluation of the HPLC method were obtained from the local central laboratory. Control sera from 132 healthy social drinkers (72 men and 60 women; age range, 24–63 years; mean age, 43 years) to be used for establishing reference intervals were obtained from workplaces in Stockholm, Sweden. Employees who were scheduled for a regular health examination at the company health service were given the opportunity to undergo a voluntary alcohol screening with the Alcohol Use Disorders Identification Test (AUDIT) *(19)* and by serum CDT measurement. Those who screened negative on the AUDIT (i.e., a score <8 for men and <6 for women) *(20)* and had no indication of excessive drinking in the alcohol consumption subscale (questions no. 1–3) *(21)* were used for this study. Their alcohol consumption was not further recorded.

For comparison, serum samples were collected from 14 healthy teetotalers (4 men and 10 women; age range, 19–69 years; mean age, 42 years) with no history of excessive drinking, all being members of a local temperance society. Serum was also collected from 122 consecutive patients (96 men and 26 women; age range, 20–72 years; mean age, 48 years) admitted for treatment of alcohol-related problems at the Center for Dependency Disorders in Stockholm. According to self-reports with a quantity–frequency questionnaire *(22)*, they had been drinking regularly (mean, 5.5 days/week and 19.7 days/month) until 1–3 days before blood sampling, which was typically performed within 24 h of admission. The mean daily alcohol intake ranged from 15 to 780 g (mean, ~200 g), and there was no significant difference ($P = 0.237$) between men (mean, 208 g) and women (166 g). In addition, serum was obtained from 21 patients (15 men and 6 women; age range, 31–65 years; mean age, 48 years) in withdrawal treatment who reported total abstention from alcohol in the last weeks before blood sampling. The vast majority of them had not drunk any alcohol or, in a few cases, had drunk small occasional amounts in the last month, according to self-reports. This was supported by negative breath ethanol and urinary 5-hydroxytryptophol *(23)* tests taken at random in connection with routine outpatient treatment visits at the clinic (data not shown).

The blood samples were collected by venipuncture in Vacutainer serum tubes. Serum was separated by centrifugation and stored at 4 °C, when analyzed within 1 day, or at −20 °C for longer times, conditions under which CDT is reported to be stable *(24)*. Frozen samples were thawed overnight at 4 °C and centrifuged at 3500*g* for 5 min before analysis. The procedures followed were approved by the ethics committee at the Karolinska Hospital and were in accordance with the Helsinki Declaration.

CHEMICALS

Dextran sulfate sodium salt and CNBr-activated Sepharose 6B were obtained from Amersham Biosciences; nitrilotriacetic acid trisodium salt monohydrate (NTA) was from Fluka; bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis-Tris), calcium chloride dihydrate ($CaCl_2 \cdot 2\,H_2O$), and iron(III) chloride hexahydrate ($FeCl_3 \cdot 6\,H_2O$) were from Sigma-Aldrich (Stockholm, Sweden); bilirubin was from VWR International; and rabbit anti-transferrin antibodies (Q327) were from Dakopatts A/S. The hemoglobin was prepared as published previously *(25)*. All other chemicals were of analytical grade, and the water was of HPLC grade (>18 MΩ).

The ferric nitrilotriacetic acid (FeNTA) solution (10 mmol/L) used for iron saturation of serum transferrin was prepared by dissolving 275 mg of NTA and 270 mg of $FeCl_3$ in 90 mL of water. The pH was adjusted to 7.0 with 1.0 mol/L NaOH, and water was added to give a final volume of 100 mL. The FeNTA solution was stored at 4 °C and is stable for at least 1 year.

The lipid precipitation solution was prepared by mixing equal volumes of 20 g/L dextran sulfate and 1.0 mol/L $CaCl_2$. The solution was stored at 4 °C and is stable for at least 1.5 months.

Anti-transferrin antibodies were bound to the CNBr-activated Sepharose 6B according to the manufacturer's instructions. Equilibration of the antibody column and elution of serum (monitored at 280 nm) were done with 0.1 mol/L Tris buffer, pH 7.5, containing 0.5 mol/L NaCl. The transferrin-free serum sample was dialyzed against 20 mmol/L Tris buffer, pH 7.5, containing 0.15 mol/L NaCl and concentrated based on the difference in albumin concentration before and after the chromatography. Regeneration of the column (i.e., elution of bound transferrin) was done with a 0.2 mol/L glycine buffer, pH 2.2, containing 0.5 mol/L NaCl. Bilirubin was dissolved in 0.1 mol/L NaOH and diluted with equal parts of water before use.

SAMPLE TREATMENT

Transferrin in the serum sample was completely saturated with iron by mixing 100 μL of serum with 20 μL of FeNTA (final concentration, 1.67 mmol/L), a well-known transferrin iron donor *(26)*. Each transferrin molecule can bind a maximum of two iron ions, but typically, serum transferrin is only partially (~30%) saturated. Because the FeNTA complex gives almost instant iron saturation of transferrin, no additional incubation time was necessary at this stage. Thereafter, the lipoproteins in the serum sample were precipitated by addition of 20 μL of the dextran sulfate–$CaCl_2$ solution. After gentle mixing, the samples were left in the cold (4 °C) for 30–60 min and then centrifuged at 3500*g* at 5 °C for 5 min. Of the clear supernatant, 100 μL was diluted with 400 μL of water and transferred to glass HPLC vials.

HPLC CONDITIONS

We routinely injected 200 μL of the pretreated serum samples into the HPLC system, which consisted of an Agilent 1100 Series Liquid Chromatograph, equipped with a quaternary pump and degasser, thermostated autosampler and column compartment, multiple wavelength detector, and ChemStation software. Separation of the transferrin glycoforms was performed on a SOURCE® 15Q PE 4.6/100 anion-exchange chromatography column (Amersham Biosciences) at ambient temperature (~22 °C) or 25 °C when thermostated, by linear salt gradient elution (Table 1) at a flow rate of 1.0 mL/min. Buffer A consisted of 10 mmol/L Bis-Tris, adjusted to pH 7.0 with 2 mol/L HCl; buffer B was the same buffer containing 0.5 mol/L NaCl, pH 6.2; and buffer C was 10 mmol/L Bis-Tris, pH 6.2. After every sample injected, the column was regenerated and cleaned by washing with 2.0 mol/L NaCl (buffer D) and finally reequilibrated with the starting buffer. All buffers were degassed and filtered through a 0.45 μm filter before use. The total run time under these conditions was ~40 min. More rigorous cleaning of the analytical column was done after every 150–200 samples, according to the manufacturer's instruction in the package insert. At least 700 analyses could be done with the analytical column without any major reduction in capacity or sensitivity.

Quantification of the transferrin glycoforms relied on the selective absorbance of the iron–transferrin complex at ~470 nm *(27)*. With the HPLC method, the relative amount of any single glycoform (e.g., disialotransferrin) or combination of glycoforms to total transferrin (all glycoforms) could be measured in terms of percentage of the area under curve (%AUC). Baseline integration was used for all transferrin peaks (from disialotransferrin, or monosialotransferrin whenever measurable, to hexasialotransferrin), as demonstrated in Fig. 1, because the results obtained in this way were less sensitive to variations in peak resolution than with valley-to-valley integration. In a pilot experiment aimed to simulate the effect of the reduced chromatographic resolution appearing over time with any column, the salt gradient slope was made

Table 1. Gradient profile used for the separation of transferrin glycoforms with the HPLC method.

Time, min	Buffer A,[a] %	Buffer B, %	Buffer C, %	Buffer D, %
0.00	100	0	0	0
1.00	100	0	0	0
1.01	0	0	100	0
30.00	0	20	80	0
30.01	0	0	0	100
35.00	0	0	0	100
35.50	100	0	0	0
37.00	100	0	0	0

[a] Buffer A, 10 mmol/L Bis-Tris, pH 7.0; buffer B, 10 mmol/L Bis-Tris + 0.5 mol/L NaCl, pH 6.2; buffer C, 10 mmol/L Bis-Tris, pH 6.2; buffer D, 2.0 mol/L NaCl.

gradually steeper, producing a gradually decreasing separation between disialo- and trisialotransferrin (data not shown). With the valley-to-valley integration mode, this gave a gradually smaller %AUC for disialotransferrin (final value, 75% of original) but not with baseline integration (103% of original). In addition, baseline integration gave higher and probably more correct values, at least for the minor transferrin glycoforms.

STATISTICS

The χ^2 test was used to test for gaussian distribution. Comparison between groups was made with the *t*-test or Wilcoxon test. Correlations were carried out with Pearson correlation coefficients. The statistical calculations were carried out using MedCalc software. $P < 0.05$ was considered significant.

Results

CHROMATOGRAPHIC SEPARATION

The HPLC system provided overall good separation between the different iron-saturated glycoforms of serum transferrin. Most importantly, disialo- and trisialotransferrin were almost baseline separated [resolution factor (R_s) ≥1.2]. A typical HPLC elution profile obtained with a serum sample from a control individual is shown in Fig. 1A. The peaks representing disialo- (retention time ~16 min), trisialo- (~18 min), tetrasialo- (~21.5 min), pentasialo- (~24 min), and hexasialotransferrin (~26.5 min) were readily identified from their respective retention times and characteristic positions in the chromatogram, with reference to previous HPLC, IEF, and structural studies *(4, 8)*. When transferrin in this serum sample was immunosubtracted with rabbit anti-human transferrin antibody, no peaks were detectable in the HPLC chromatogram (Fig. 1A, inset). This was also true for sera with measurable asialo- and monosialotransferrin (data not shown), thus confirming that all peaks observed in the chromatogram at 470 nm were indeed transferrin glycoforms. Furthermore, this experiment excluded any interference of the heme-binding hemopexin, which usually comigrates with transferrin in conventional electrophoresis.

A typical HPLC elution profile for a serum sample from a chronic alcohol consumer is shown in Fig. 1B. In addition to the alcohol-related increase in disialotransferrin, an early-eluting peak (retention time ~10 min) representing asialotransferrin was measurable. However, asialotransferrin was detected only in cases with high disialotransferrin (~2% and higher). In some sera showing a high relative amount of trisialotransferrin (>6%), a peak representing monosialotransferrin (~14 min) was measurable (Fig. 1C), eluting immediately ahead of disialotransferrin in the chromatogram.

HPLC chromatograms of sera for the most common genetic transferrin variants obtained from the local hospital central laboratories, corresponding to transferrin BC, CD, and "C2C3" heterozygotes *(3)*, are shown in Fig. 1, D–F. Additionally, several very rare variants, including the transferrin B homozygote, have been identified over the years (data not shown).

SENSITIVITY AND LINEARITY

The limit of detection in %AUC for the transferrin glycoforms (determined for asialo- and monosialotransferrin) was ~0.05% of total transferrin, but in routine use a limit of quantification of ~0.1% was applied. It should be noted that the limit of detection and limit of quantification depend in part on the transferrin concentration in the serum sample, which can be determined by routine clinical chemistry methods but also estimated from the total AUC for all transferrin glycoforms in the HPLC chromatogram *(28)*. Because all glycoforms except tetrasialotransferrin occur at low concentrations *(29)* and the absorbance of the transferrin–iron complex at 470 nm is only ~10% compared with the absorbance at 280 nm *(8)*, the performance of the photometric detector is also very important.

When a serum sample containing a very high transferrin concentration (~5 g/L; reference interval, 1.9–3.3 g/L) was diluted with water before sample treatment to 75%, 50%, 25%, and 10% of the original concentration (i.e., tested range ~0.5–5 g/L), the AUC for total transferrin was linear ($r^2 = 0.999$). Furthermore, the relative amount of disialotransferrin was not markedly influenced (CV = 6.0%). The total transferrin AUC was linear with injection volumes of 50–250 μL ($r^2 = 0.999$; mean R_s, 1.29; R_s range, 1.22–1.37), and the relative amount of disialotransferrin was not different (CV = 2.7%).

REPRODUCIBILITY

Data on the reproducibility of the HPLC method for measurement of the relative amounts of disialotransferrin and asialotransferrin with serum samples from controls and heavy drinkers are given in Table 2. The within-day (intraassay) and between-day imprecision (CV) for serum samples containing disialotransferrin values in the range 1–5.6% were <5%. At the low concentrations of asialotransferrin examined (~0.3%), the intraassay CV was >10%. The retention times for the transferrin glycoforms were stable over time, the within- and between-day CV for disialotransferrin being ~0.3% and ~1.3%, respectively (n = 10).

INTERFERENCES

Identical results were obtained when the serum samples were mixed with FeNTA at final concentrations of 0.17, 0.33, 0.84, and 1.67 mmol/L (CV = 0.7% for the total transferrin AUC and 5.7% for the relative amount of disialotransferrin). To study the effect of incomplete iron saturation of transferrin, we prepared the serum sample for HPLC analysis without addition of FeNTA. This produced a very different HPLC profile, with new peaks appearing to the left in the chromatogram (Fig. 2, A and B). This occurs because the charge of the transferrin

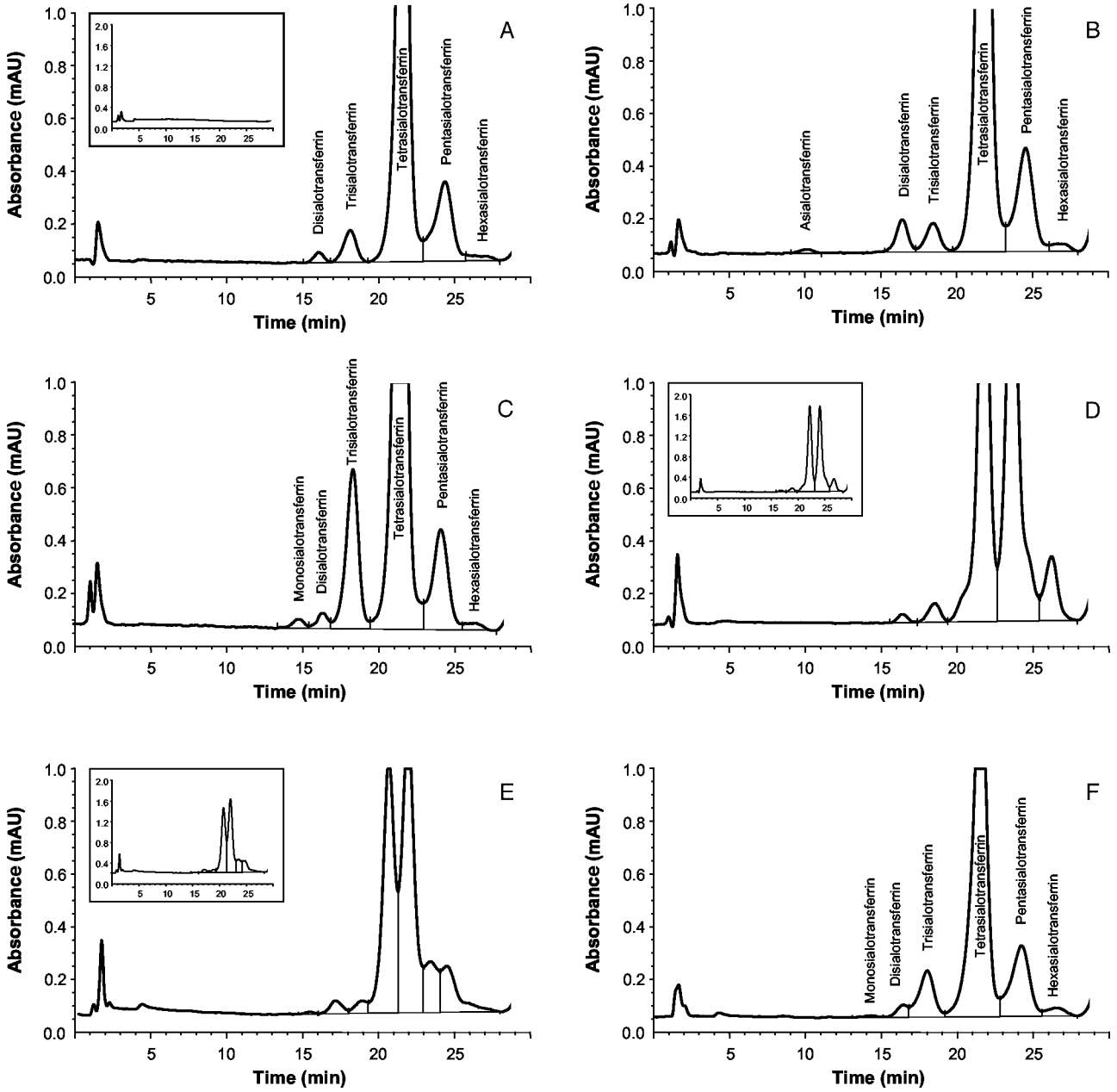

Fig. 1. Transferrin glycoform patterns for different serum samples with the HPLC method.

HPLC chromatograms were obtained for the following samples: (*A*), a control serum sample from a light drinker with the predominant transferrin C homozygote variant (*inset*, the same sample after immunosubtraction of transferrin); (*B*), transferrin C serum from a heavy drinker with increased disialotransferrin (3.2% of total AUC for transferrin) and detectable (~0.4%) asialotransferrin; (*C*), transferrin C serum from a person with high trisialotransferrin (8.4%) and a measurable (~0.5%) monosialotransferrin; (*D*), transferrin BC heterozygote (*inset*, the same chromatogram at full scale); (*E*), transferrin CD heterozygote (*inset*, the same chromatogram at full scale); and (*F*), transferrin C2C3 variant.

molecule differs depending on the amount of iron saturation, and the nonsaturated (Fe_0) or partially saturated [transferrin saturated at the N-terminal (Fe_{1N}) or C-terminal (Fe_{1C}) domain] glycoforms will thus elute differently from the completely iron-saturated (Fe_2) forms *(17)*. However, the high reproducibility of test results observed when measurements were carried out under standard conditions (Table 2) indicated that the transferrin iron load was complete and stable, with no risk for loss of iron during the chromatographic separation.

The HPLC method was also tested for use with samples of plasma. With lithium heparin and sodium heparin/fluoride plasma, the HPLC method allowed for separation and measurement of all transferrin glycoforms, similar to the results observed with serum. However, with EDTA plasma, a high, asymmetric peak was always observed at about the same retention time as for asialotransferrin (Fig. 2C). This peak was also observed with blank samples (EDTA tube containing water), confirming that it was an artifact peak and not the result of iron

Table 2. Reproducibility of the relative amounts of disialo- and asialotransferrin to total transferrin in serum with the HPLC method.

Serum sample	n	Mean (range) disialotransferrin, %	CV, %	Mean (range) asialotransferrin, %	CV, %
Control individuals					
Intraassay	5	1.03 (0.99–1.09)	4.6	ND[a]	
Interassay	5	1.05 (0.98–1.10)	4.5	ND	
Interassay	153[b]	1.07 (0.98–1.21)	4.4	ND	
Heavy drinkers					
Intraassay	5	2.12 (2.03–2.21)	3.6	ND	
Intraassay	5	5.58 (5.37–5.84)	3.1	0.32 (0.26–0.36)	12
Interassay	5	2.07 (2.00–2.13)	2.6	ND	
Interassay	157[b]	2.12 (2.00–2.29)	2.9	ND	

[a] ND, not detected (limit of detection, 0.05%).
[b] Measurements were carried out over 13 months.

removal from transferrin by complexation with EDTA. With citrate plasma, an extra peak that partially coeluted with tetrasialotransferrin was observed (Fig. 2D). This peak was not observed with blank citrate samples.

Analysis of control samples with and without dextran sulfate and $CaCl_2$ showed results very similar to those obtained with the HPLC method. Bilirubin and hemoglobin were added in concentrations up to 150 μmol/L and 0.7 g/L, respectively, without any marked interference with the quantification of transferrin glycoforms. Furthermore, we observed no interference of C-reactive protein (CRP) at concentrations up to 242 mg/L.

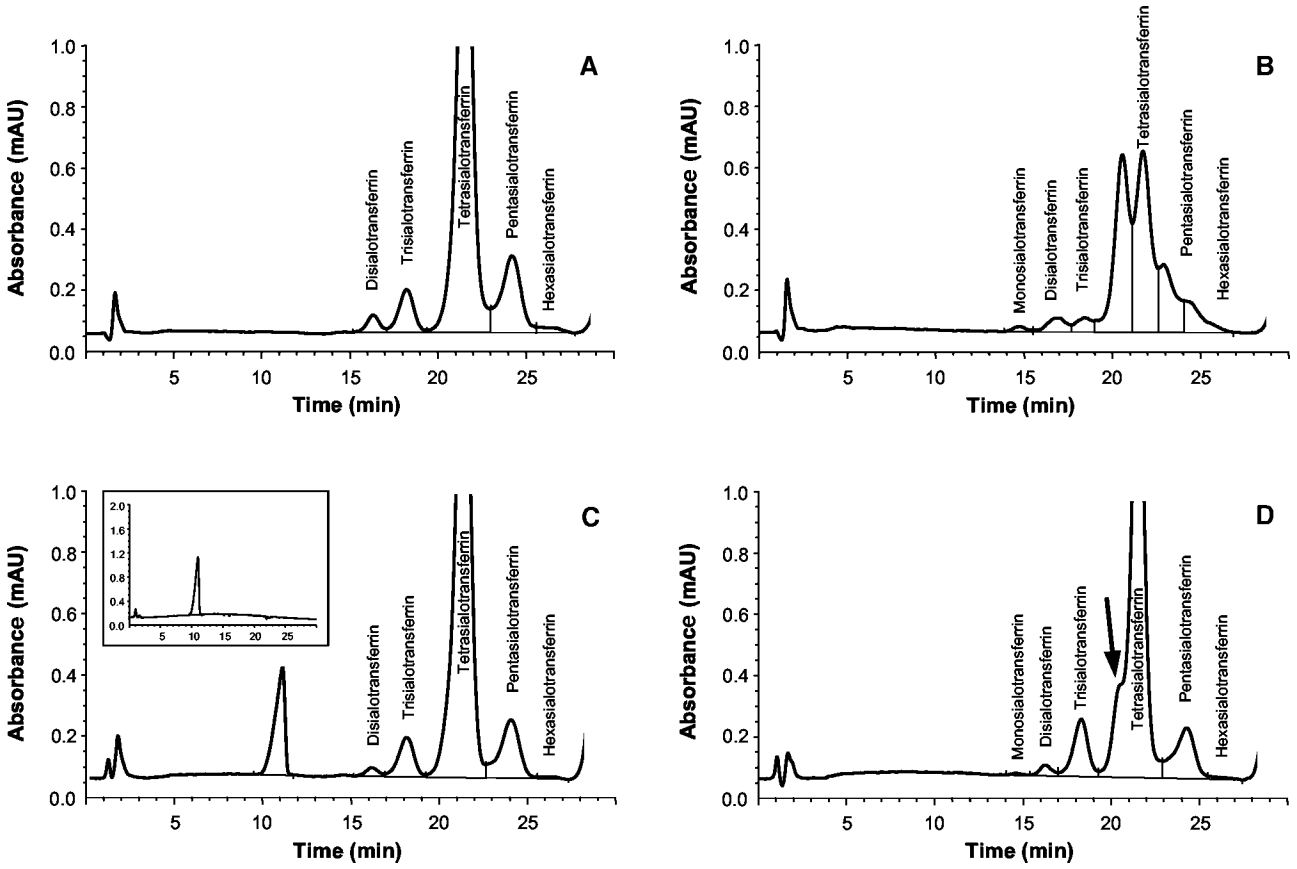

Fig. 2. Transferrin glycoform patterns for serum and plasma samples with the HPLC method.

HPLC chromatograms obtained for the following samples: (*A*), transferrin C serum from a heavy drinker with increased disialotransferrin (2.1%); (*B*), the same serum sample as in *A* but without treatment with FeNTA (i.e., no complete iron saturation of transferrin); (*C*), EDTA plasma from a light drinker showing a typical asymmetric peak eluting at ~11 min (*inset*, EDTA blank sample); and (*D*), citrate plasma from a light drinker showing an additional peak (*arrow*) partially coeluting with tetrasialotransferrin.

Table 3. Stability of relative amounts of asialo-, disialo-, and monosialotransferrin to total transferrin in serum samples and the same samples after pretreatment for HPLC analysis under different storage conditions.

CV, %

	Control serum		Alcohol serum					
			Man			Woman		
Storage conditions	Man (Disialotransferrin ~1.1%)[a]	Woman (Disialotransferrin ~1.3%)	Asialotransferrin (~0.5%)	Monosialotransferrin (~0.6%)	Disialotransferrin (~5.5%)	Asialotransferrin (~0.3%)	Disialotransferrin (~6.0%)
Serum at 4 °C for 0, 1, 3, and 7 days	4.3[b]	6.2	9.9	13	2.5	12	3.6
Serum at 22 °C for 0, 1, 3, and 7 days	6.0	4.7	7.8	5.4	0.7	14	2.5
Serum at −20 °C for 0, 1, and 3 months	1.5	2.8	18	8.5	2.2	31	0.9
Freezing at −20 °C and thawing of serum in 0, 1, 2, and 3 cycles	1.7	4.4	14		1.5	9.0	0.9
Pretreated sample at 4 °C for 0, 12, 24, and 48 h	6.0	4.6	18		1.8	12	1.0
Pretreated sample at 22 °C for 0, 12, 24, and 48 h	5.3	1.6	16		0.8	3.1	0.9

[a] Values in parentheses indicate the relative amounts of transferrin glycoforms examined (limit of quantification, ~0.1%).
[b] Results of single determinations.

STABILITY OF SAMPLES

The stabilities of the relative amounts of asialotransferrin and disialotransferrin in serum samples from two females and two males (one control individual and one heavy drinker of each gender) and in the same samples after pretreatment for HPLC analysis under different conditions are shown in Table 3. Both the sera and pretreated samples were relatively stable on storage at room temperature or in the cold for days as well as during typical handling in the laboratory (repeated freezing and thawing of serum).

REFERENCE INTERVALS FOR TRANSFERRIN GLYCOFORMS

The relative distributions of %AUC for the different transferrin glycoforms to total transferrin in sera from 132 healthy controls are given in Table 4. Tetrasialotransferrin was the predominant glycoform, accounting for ~80% on average, followed by pentasialo- and trisialotransferrin. Monosialotransferrin was detected at low concentrations (<0.25%) in some cases of a high trisialotransferrin (>6%), whereas asialotransferrin was not measurable in any of the control sera. Small but significant ($P < 0.05$) gender differences were observed in the relative amounts of trisialo- (women > men), tetrasialo- (men > women), and the sum of pentasialo- and hexasialotransferrin (women > men; Table 4).

The distribution of disialotransferrin values, the major CDT glycoform, in the serum samples collected from healthy social drinkers was compared with the results for 14 healthy nondrinkers (teetotalers), 122 consecutive patients admitted for treatment of alcohol- and drug related problems, and 21 patients undergoing treatment who had abstained from drinking in the last weeks before blood sampling according to self-reports (Fig. 3). The control population showed disialotransferrin values in the range 0.49–1.77% [mean, 1.16%; 95% confidence interval (CI), 1.12–1.20%; 2.5th–97.5th percentiles, 0.67–1.67%; Fig. 4], and this compared with 0.66–1.56% (mean, 1.01%; 95% CI, 0.87–1.14%; $P = 0.055$) for the nondrinkers and 0.56–2.20% (mean, 1.13%; 95% CI, 0.95–1.32%; $P = 0.866$) for the alcohol-abstinent patients (Fig. 3). The patients in withdrawal treatment who admitted drinking alcohol in the last week (range, 15–780 g/day on average) showed values for disialotransferrin between 0.56% and 16.35% (mean, 4.25%). Asialotransferrin was detected at 0.15–4.38% of total transferrin in 57% of chronic heavy drinkers (at least 50 g/day on 4 or more days in the last week according to self-report; n = 83) and in 62% of the sera with a disialotransferrin ≥2% (n = 85).

Discussion

The HPLC system provided reproducible separation and quantification of the asialo-, monosialo-, disialo-, trisialo-, tetrasialo-, pentasialo-, and hexasialotransferrin glycoforms, as identified from their retention times and relative positions in the chromatogram with reference to previous studies *(4, 8)*. Most importantly, disialo- and trisialotrans-

Table 4. Relative distributions of the different transferrin glycoforms in %AUC to total transferrin in sera from 132 healthy controls.

	Monosialotransferrin	Disialotransferrin	Trisialotransferrin	Tetrasialotransferrin	Penta- + Hexasialotransferrin
All samples (n = 132)					
Mean (SD), %	0.01 (0.03)	1.16 (0.25)	4.77 (1.36)	80.18 (2.01)	13.88 (1.69)
95% CI for the mean, %	0.00–0.01	1.12–1.20	4.53–5.00	79.83–80.52	13.59–14.17
Median, %	0.00	1.17	4.53	80.38	13.52
Range, %	0–0.24	0.49–1.77	2.61–10.53	70.82–83.60	9.96–19.90
χ^2		4.38	14.95	19.77	19.64
P		0.976[a]	0.134[a]	0.032[b]	0.050[a]
Women (n = 60)					
Mean (SD), %	0.02 (0.05)	1.15 (0.25)	5.08 (1.51)	79.44 (2.40)	14.31 (2.07)
Men (n = 72)					
Mean (SD), %	0.00 (0.01)	1.17 (0.25)	4.50 (1.17)	80.79 (1.34)	13.53 (1.20)
Wilcoxon, *P*	0.393	0.527	0.021[c]	0.001[c]	0.043[c]

[a] Accept normality.
[b] Reject normality.
[c] Significant difference between results for men and women.

ferrin were almost baseline separated, which represents a major improvement over previous HPLC methods for quantification of CDT *(8, 14, 30, 31)*. Because the trisialotransferrin concentration usually exceeds the disialotransferrin concentration by approximately fourfold (see Table 4), partial coelution of these neighboring peaks may otherwise cause overestimation of the true amount of disialotransferrin, the major CDT glycoform, and hence increase the risk for false-positive identification of alcohol abuse *(17)*. The HPLC method did not allow for baseline separation between disialo- and trisialotransferrin in serum containing a rare genetic transferrin variant, tentatively identified as C2C3 *(3, 32)*. Nonetheless, genetic transferrin variants, including the most common BC, CD, and C2C3 heterozygotes, were readily identified from a unique peak pattern in the chromatograms.

The relative distribution of transferrin glycoforms obtained in this study, using control sera from social drinkers, was in agreement with the results of a previous HPLC study that used patient samples with a "normal" CDT (i.e., disialotransferrin) concentration obtained from the routine analysis *(33)*. Accordingly, tetrasialotransferrin typically accounted for ~80% of all transferrin in human serum, whereas trisialo- and pentasialotransferrin accounted for ~5% and ~10–15%, respectively, and disialotransferrin for ~0.5–2%. Small but statistically significant gender differences were observed for the higher glycoforms but not for disialotransferrin. The use of

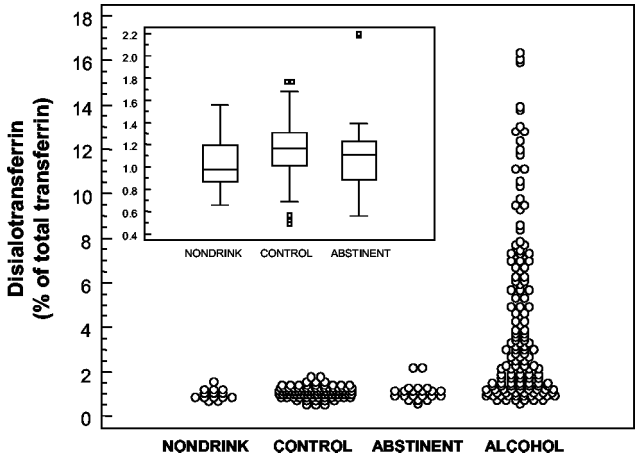

Fig. 3. Distribution of relative amounts of disialotransferrin (%AUC) in sera from different populations.

Serum samples were collected from 14 healthy teetotalers, 132 healthy social drinkers, and 143 consecutive patients admitted for treatment of alcohol-related problems, 21 of whom had abstained from drinking alcohol in the last weeks before sampling, according to self-reports. (*Inset*), box-and-whisker plots (median, 25th–75th percentiles, and minimum and maximum values with outside values displayed as separate *points*) for the teetotalers, social drinkers, and abstinent patients separately.

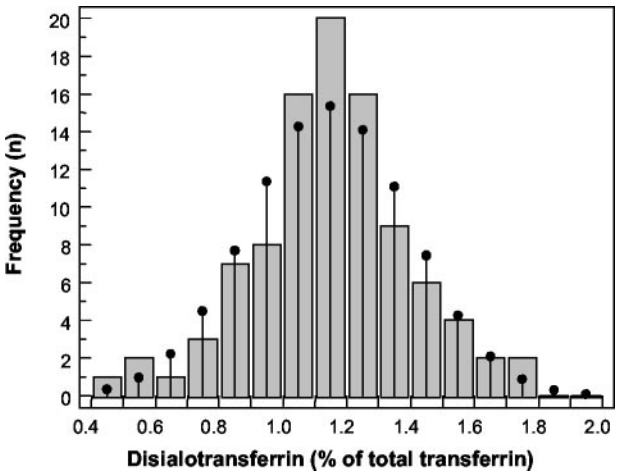

Fig. 4. Distribution of the relative amounts of disialotransferrin (%AUC) in sera from healthy controls.

Serum samples were collected from 132 healthy social drinkers in connection with a regular health examination at the company health service. Only those who screened negative on the AUDIT (score <8 for men and <6 for women) and had no indication of excessive drinking were included in this study. Range, 0.49–1.77%; mean (SD), 1.16 (0.25)%; median, 1.17%. The *vertical lines* represent the theoretical gaussian distribution.

baseline integration from disialotransferrin through hexasialotransferrin (see example in Fig. 1A), instead of valley-to-valley integration as originally described *(8)*, yielded constantly higher and probably more correct values for the minor glycoforms. In this context, the mean (SD) disialotransferrin values obtained [1.16 (0.25)%] concur with the results of another HPLC study of healthy blood donors that also used the baseline integration mode [1.13 (0.27)%] *(14)*. Monosialotransferrin was measurable only in some samples with high trisialotransferrin (>6%) *(3)*, and in those cases the baseline integration should also cover this peak (see Fig. 1C).

As demonstrated previously *(3, 33)*, the asialotransferrin peak was detected only in cases of increased disialotransferrin (~2% and higher). Therefore, based in part on studies of chronic heavy drinkers *(34)*, it has been proposed that a measurable asialotransferrin would represent a sensitive and specific single indicator of alcohol abuse *(35)*. However, because asialotransferrin was not detected in all samples with an increased disialotransferrin in the present study, this analytical strategy might suffer from a lower diagnostic sensitivity. This is further supported by the observation that significant increases in asialotransferrin were observed only in "chronic" but not in "high" alcohol consumers, whereas disialotransferrin was significantly increased in both these groups *(29)*.

In addition to HPLC, CE has also been used for the visible separation and quantification of transferrin glycoforms in detection of chronic alcohol misuse. In recent years, several CE methods with improved separation efficiency between the glycoforms have been published *(15, 16, 36)*. The usual concentration of disialotransferrin obtained by CE (mean value, ~0.5%) *(36)* is considerably lower than that reported in the present and previous HPLC studies (mean, ~1.2%). This suggests that the current CE methods may not be as sensitive for quantification of the minor glycoforms (e.g., disialotransferrin), despite a reported detection limit of 0.03% of total transferrin. However, the lower concentrations could also be attributable in part to the use of valley-to-valley integration *(36)*. A potential problem with the CE method, which uses detection at 214 nm, where all proteins absorb, is that CRP interferes with the measurements by coeluting with the monosialotransferrin peak in the electropherograms *(36)*. In contrast, no interference of CRP was seen with the quantification of transferrin glycoforms by HPLC, at very high CRP concentrations, because this method relies on the selective absorbance of the iron–transferrin complex at ~470 nm *(27)*. Further studies are warranted to compare the analytical performance and routine applicability of the HPLC and CE methods.

The HPLC method showed good agreement of results both within and between assays over a long time (>1 year). In agreement with previous observations *(24)*, this study suggested that serum transferrin is fairly stable on storage. The samples could be stored for days at room temperature or in the cold, and for months in the freezer, without any marked change in results. In contrast, other studies have reported on the risk for falsely high CDT results with immunoassays when serum samples were stored improperly for several days *(10, 37)*. This might possibly result from samples being contaminated with microbes that produce neuraminidase (sialidase), because this will lead to the successive removal of terminal sialic acid residues from the glycans (i.e., peaks shifted to the left in the HPLC chromatogram) and, hence, a gradually higher CDT result with the immunoassays *(3)*. Although the atypical transferrin isoform pattern observed by HPLC after serum was treated with neuraminidase *(3)*, or in "aged" samples *(30)*, is easily distinguishable from the alcohol-selective increases of disialotransferrin and, in cases of a high disialotransferrin, asialotransferrin, storage of serum samples in the cold should always be recommended. The pretreated serum sample was stable for at least 2 days at room temperature or in the cold. Accordingly, the method allows for overnight analytical runs, even when the HPLC system is not equipped with a thermostated autoinjector, and the sample volume is also sufficient for one reanalysis.

In conclusion, with respect to simplicity and overall analytical performance, the HPLC method appeared to fulfill the requirements of a qualitative and quantitative preliminary reference method for the relative distribution of transferrin glycoforms in serum. Accordingly, the method should work for any combination of glycoforms covered by the present and any future CDT methods used for detecting high alcohol consumption. Use of a reference method could improve the reliability of routine CDT analysis and comparability of results between studies using different methods. A drawback is the lack of commercially available and well-characterized transferrin glycoform standards. When such calibrators are available, this method may become part of a reference system for CDT. The HPLC method could also be useful for confirmatory analysis of positive CDT results by immunoassays in medico-legal cases, to exclude the risk for false-positive identification of alcohol abuse as a result of genetic transferrin variants *(3, 18)*. For such applications, the total run time of the assay may be reduced by applying a steeper salt gradient.

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