

# Determination of Carbohydrate-deficient Transferrin Using Capillary Zone Electrophoresis

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**Background:** Current methods for carbohydrate-deficient transferrin (CDT) often suffer from low precision, complexity, or risk of false positives attributable to genetic variants. In this study, a new capillary zone electrophoresis (CZE) method for CDT was developed.

**Methods:** CZE was performed on a P/ACE 5000 using fused-silica capillaries [50  $\mu\text{m}$  (i.d.)  $\times$  47 cm] and the CEOFIX CDT buffer system with addition of 50  $\mu\text{L}$  of anti-C3c and 10  $\mu\text{L}$  of anti-hemoglobin. Native sera were loaded by high-pressure injection for 3 s, separated at 28 kV over 12 min, and monitored at 214 nm.

**Results:** CDT was completely resolved by differences in migration times (di-trisialotransferrin,  $9.86 \pm 0.05$  min; monosialotransferrin,  $9.72 \pm 0.05$  min; asialotransferrin,  $9.52 \pm 0.04$  min), with a CV of 0.15%. The number of theoretical plates was  $312\,000 \pm 21\,000$  for the mono- and  $199\,000 \pm 6500$  for the di-trisialylated transferrin. Genetic CB and CD variants showed prominent peaks with migration times of  $10.12 \pm 0.06$  and  $9.89 \pm 0.03$  min, respectively, and the carbohydrate-deficient glycoprotein syndrome could be detected, excluding false-positive results. CZE results (as a percentage;  $y$ ) correlated with the Axis %CDT TIA<sup>TM</sup> ( $x$ ) values by Deming regression analysis:  $y = 1.92x - 7.29$ ;  $r = 0.89$ . CDT values in 130 healthy nonalcoholics were determined. The 2.5th and 97.5th percentiles were 1.84% and 6.79%. **Conclusions:** CZE without sample pretreatment can determine CDT with good precision, allows detection of vari-

ants, and correlates with ion-exchange chromatography.

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Alcohol is the most frequently abused drug throughout the world, leading not only to irreversible physical injuries, neuropsychiatric defects, social problems, and trauma, but also to substantial costs for society (1). Conventional laboratory tests for  $\gamma$ -glutamyltransferase (EC 2.3.2.2) and mean corpuscular volume have low diagnostic sensitivity for detecting alcohol abuse before the stage of organic complications and are indicators of disease in a particular organ, with poor specificity for different etiological possibilities (2–4).

Since the discovery that alcoholics demonstrate an abnormal transferrin electrophoretic pattern (5), carbohydrate-deficient transferrin (CDT)<sup>6</sup> has been considered the most valuable marker for monitoring chronic alcohol abuse (2). Transferrin exhibits a microheterogeneity in its iron, amino acid, and carbohydrate content. Variations in iron content can be overcome by saturating samples with iron (6), and amino acid variations are seen only in rare genetic variants of transferrin (2). The commonly occurring isoforms of diferric transferrin depend on variations in carbohydrate content. Normal serum contains high concentrations of tetrasialotransferrin (pI 5.4) and low amounts disialo-, trisialo-, and pentasialotransferrin (pI 5.7, 5.6, and 5.2, respectively) (2, 6, 7). In alcoholics, transferrin fractions with di- (pI 5.7), mono- (pI 5.8), and asialylated (pI 5.9) carbohydrate chains are increased (8). It has been estimated that a minimum alcohol consumption of 50–80 g/day for at least 1 week is needed to increase the serum CDT in ~80% of subjects. The half-life of the marker is 16–17 days (2, 9, 10).

Early methods for CDT used isoelectric focusing (IEF) with laser densitometric quantification after immunoblotting (11). However, although this technique served as

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<sup>6</sup> Nonstandard abbreviations: CDT, carbohydrate-deficient transferrin; IEF, isoelectric focusing; CZE, capillary zone electrophoresis; and CDG, carbohydrate-deficient glycoprotein syndrome.

gold standard for the validation of most CDT procedures, IEF was too complex and laborious for routine clinical purposes. Alternative procedures were introduced based on anion-exchange chromatography (2, 12), chromatofocusing (13), HPLC (8) and fast protein liquid chromatography (14), but they also were complex and laborious. Recently, commercial methods for easy, time-saving CDT detection have been developed that use anion-exchange chromatography followed by RIA (CDTect™ and %CDT-RIA™), enzyme immunoassay (CDTect-EIA™), or turbidimetric immunoassay (%CDT-TIA™ and ChronAlcoI.D.™).

In 1989, capillary IEF was successfully applied to quantitative measurement of CDT (15). However, this method is not easily applied in a routine clinical environment. The problems in separation of transferrin isoforms using capillary zone electrophoresis (CZE) (16–18) could be resolved by an improved CZE method requiring only iron saturation as sample pretreatment (19–21). This opened prospects for application in a routine clinical laboratory.

In this study, we describe a new CZE method using the CEOFIX CDT buffer system (Analisis, Namur, Belgium), which requires no sample pretreatment. Furthermore, influence of the presence of transferrin variants and carbohydrate-deficient glycoprotein syndrome (CDG) on the CDT analysis was evaluated. Finally, this method was compared with an ion-exchange chromatography-based method (%CDT-TIA; Axis Biochemicals).

### Materials and Methods

#### SUBJECTS

Reference serum samples were collected from 130 healthy subjects (69 males, age  $41 \pm 16$  years; 61 females, age  $35 \pm 15$  years) who showed no biochemical or clinical evidence of chronic alcohol abuse (social drinkers were included). The comparison study with an ion-exchange chromatographic assay was performed on serum samples from 77 (60 males, age  $45 \pm 9$  years; 17 females, age  $47 \pm 10$  years) nonalcoholic, healthy subjects (social drinkers were included) and patients admitted to the Department of Psychiatry of the AZ Stuivenberg Hospital with suspicion of chronic alcoholism. This population was selected to obtain a wide range of CDT values. CDT was also studied in 10 other patients (3 males, age  $48 \pm 17$  years; 7 females, age  $50 \pm 27$  years) with iron deficiency anemia, defined as mean corpuscular volume  $<80$  fL, hemoglobin  $<115$  and  $<135$  mg/L, serum ferritin  $<11$  and  $<36$   $\mu\text{g/L}$ , and serum iron  $<230$  and  $<350$   $\mu\text{g/L}$  for females and males, respectively.

Sera from heterozygous individuals carrying the CD variant of transferrin were obtained from 2 healthy Caucasians and 11 Zimbabwean Bantus, an ethnic group with a high incidence of genetic CD transferrin variance. Sera from heterozygous individuals carrying the CB variant were obtained from two healthy Caucasians. Serum from

a CDG patient was obtained from the Department of Pediatrics of the University Hospital, Gent.

#### CDT ANALYSIS

**Capillary electrophoresis.** Analysis was carried out with a P/ACE 5000 electropherograph (Beckman) with a single-wavelength ultraviolet absorbance detector and an interference filter at 214 nm. Uncoated fused-silica capillaries [ $50 \mu\text{m}$  (i.d.)  $\times$  47 cm] were purchased from Beckman. The buffer system of the CEOFIX CDT method (Analisis) was used. To 4.2 mL of running buffer, 50  $\mu\text{L}$  of anti-C3c antibodies and 10  $\mu\text{L}$  of anti-hemoglobin (Dade Behring) were added. The capillary was first rinsed for 1.2 min under pressure with the “initiator” solution, which coated the capillary wall with a polycation. This rinse was followed by a 1.5-min rinse with a “buffer” solution containing a polyanion, which adds a second layer of coating. Serum was loaded electrophoretically by pressure injection for 3 s. Separation was performed over 12 min at a constant voltage of 28 kV, and the peak areas were calculated by integration software from Beckman. Between samples, the capillary was washed with 0.2 mol/L NaOH for 1.5 min, followed by water for 1 min. Electrophoresis conditions were established for CDT analysis without any sample pretreatment.

**Turbidimetric immunoassay.** CDT was determined by the Axis %CDT turbidimetric immunoassay (%CDT-TIA) according to the manufacturer’s instructions. Briefly, serum transferrin is saturated with  $\text{Fe}^{3+}$  before the low-sialic acid transferrin (CDT) is separated by an ion-exchange chromatography minicolumn. The eluate and the total transferrin are measured using the same anti-transferrin antibodies on a Cobas Mira Analyzer (Roche Diagnostics). The %CDT value is calculated, and values exceeding 6.0% are considered increased.

#### QUANTIFICATION AND CZE PERFORMANCE CHARACTERISTICS

The peaks corresponding to transferrin isoforms were quantified as the asialo-, monosialo-, di-trisialo-, tetrasialo-, and pentasialotransferrin content as a percentage of the total transferrin content, in terms of area under the curve. In common CC transferrins, the CDT value was calculated by the addition of the peak areas (%) of the asialo-, monosialo-, and di-trisialotransferrins. In CD variants, the peak areas (%) of the asialo-D, asialo-C, monosialo-D, monosialo-C, and di-trisialo-D transferrins were used for CDT calculations. Capillary electrophoresis separation characteristics were calculated according to the United States Pharmacopoeia calculation method. Efficiency, expressed as number of theoretical plates ( $N$ ), was calculated as:  $N = 16(t/W)^2$ , where  $t$  is the migration time, and  $W$  is the peak width at the basis of the peak using tangent method. Resolution ( $R$ ) was calculated on the basis of the equation:  $R = 2(t_2 - t_1)/(W_2 - W_1)$ , where  $t_2$  and  $t_1$  represent the migration times of two adjacent

peaks, and  $W_2$  and  $W_1$  represent the widths at the baseline of these two peaks. Peak asymmetry is calculated as the tailing factor  $T = W_{0.05}/2f$ , where  $W_{0.05}$  is the distance from the leading edge to the tailing edge of the peak, measured at a point 5% of the peak height from the baseline, and  $f$  is the distance from the peak maximum to the leading edge of the peak at the position of 5% peak height. The reproducibility of the peak area percentages and migration times of the mono-, di-tri-, and tetrasialylated transferrin peaks was tested by assaying the same pathological sample 22 times.

#### RESOLUTION OF SERUM TRANSFERRIN ISOFORMS

Serum (50  $\mu$ L) was incubated overnight with 100  $\mu$ L of anti-transferrin antiserum (Behring). After centrifugation at 2000g for 20 min, the supernatant was collected and analyzed. The electropherographs of the serum before and after immunosubtraction were compared to locate the transferrin peaks among the bulk of serum proteins.

Resolution of the serum transferrin isoforms was performed by incubating 50  $\mu$ L of serum with 0.01 U of neuraminidase (type V, from *Clostridium difficile*; EC 3.2.1.18; Sigma) at 37 °C. Serum from a healthy nonalcoholic subject was analyzed without incubation and after 0.5, 1, 3, 5, and 9 h of incubation. Sera from carriers of the CB and CD variants were analyzed without and after 0.5, 1, 3, and 5 h of incubation.

Interference from the C3c degradation products of complement C3 was investigated by comparing the results for a sample from a healthy subject analyzed after >2 h at room temperature using the CEOFIX CDT buffer (Analis) with and without the addition of anti-C3c antiserum (Behring) to the running buffer.

To investigate interference from sample instability, a fresh sample was analyzed within 2 h after venipuncture and then divided into three aliquots, which were stored refrigerated (4–8 °C), at room temperature (18–22 °C), and at 37 °C. Each aliquot was analyzed daily for 5 consecutive days, and the results were compared.

Iron saturation of transferrin was performed by incubating 1 mL of serum overnight with 25  $\mu$ L of 500 mmol/L  $\text{NaHCO}_3$  and 18  $\mu$ L of 10 mmol/L  $\text{FeCl}_3$ . Serum samples from patients with iron deficiency anemia were analyzed with and without iron saturation before analysis. The capacity of on-column iron saturation through the presence of iron in the running buffer was evaluated by comparing the percentages of monosialo- and di-trisialo-transferrin values for samples from patients with iron deficiency anemia analyzed without and with iron saturation before analysis.

#### STATISTICS

Results are expressed as the mean  $\pm$  SD. Within-run imprecision was assessed by calculating the CV of the peak area percentages and migration times of the monosialo-, di-trisialo-, and tetrasialotransferrin peaks of the same pathological sample. Peak area percentage CVs

were calculated to assess the between-day variation. The effect of on-column iron saturation was evaluated by analyzing 10 samples from patients with iron deficiency anemia with and without preanalysis iron saturation. Differences in the monosialo- and di-trisialotransferrin peaks of samples saturated with iron before analysis and native samples were evaluated using the paired *t*-test. Deming regression analysis was used for the comparison study between capillary electrophoresis and Axis %CDT-TIA. The population of healthy nonalcoholic subjects was evaluated for gaussian distribution by the Kolmogorov-Smirnov test. The Student *t*-test and ANOVA were used to evaluate gender and age differences in this population.

#### Results

##### RESOLUTION AND CZE PERFORMANCE CHARACTERISTICS

The transferrin peak was identified among the bulk of serum components by an immunosubtraction experiment. The precipitation of the transferrin-anti-transferrin complex eliminated the transferrin peaks from the electropherograph, whereas the other serum proteins remained visible. This enabled identification of the transferrin peaks, preventing misinterpretation of the results.

To demonstrate the ability of the proposed method to resolve all of the CDT isoforms, serum was incubated with neuraminidase for increasing times to obtain sequential cleavage of the sialic acid moieties (Fig. 1). In the electropherograph obtained without incubation, tetrasialotransferrin (migration time,  $10.05 \pm 0.02$  min) was the dominant peak, with a moderate concentration of penta-sialotransferrin (migration time,  $10.10 \pm 0.06$  min) and a low concentration of di-trisialotransferrin (migration time,  $9.86 \pm 0.05$  min). After 30 min of incubation, a decrease in the penta- and tetrasialo peaks and an increase in the di-trisialo peak could be seen. With increasing incubation times, monosialotransferrin (migration time,  $9.72 \pm 0.05$  min) and asialotransferrin (migration time,  $9.52 \pm 0.04$  min) appeared and became the dominant peaks, whereas the penta-, tetra-, and di-trisialic forms decreased and disappeared. After 9 h of incubation, the asialylated peak became the dominant peak, and only a trace of the monosialylated peak was present.

Table 1 shows the CZE performance characteristics number of theoretical plates, resolution, and peak asymmetry. Within-run reproducibility of the absolute and relative (calculated as ratios of the migration time of the tetrasialo peak) migration times and the peak area percentages of the monosialo-, di-trisialo-, and tetrasialotransferrins from the same pathological serum are included (Table 1).

Serum stored at least 2 h at room temperature after venipuncture was analyzed with and without anti-C3c in the running buffer. In the analysis without anti-C3c, the tail of a broad additional peak (migration time,  $9.52 \pm 0.02$  min) interfered with the detection of asialotransferrin. Addition of anti-C3c antibodies to the running buffer eliminated the interfering peak, clearing the baseline of noise.

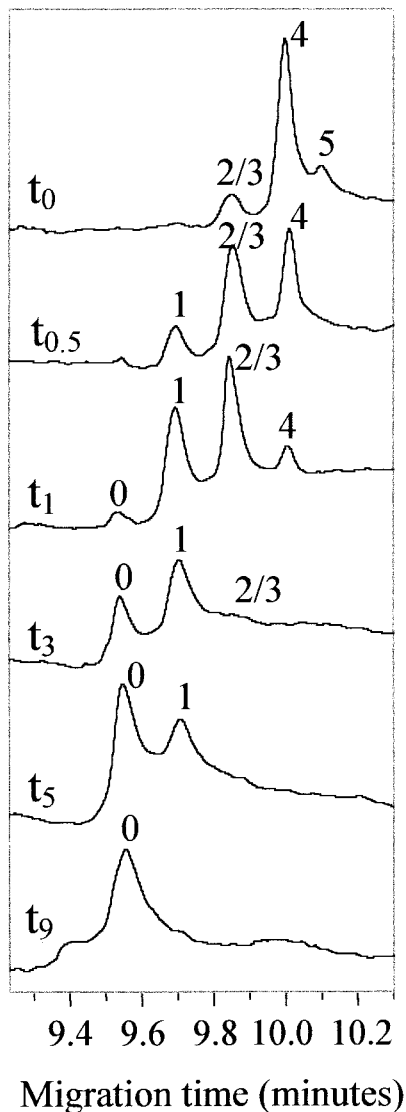


Fig. 1. Effect of neuraminidase treatment to cleave the sialic acid residues on the spectrum of glycosylated transferrin isoforms without incubation ( $t_0$ ) and after 0.5 ( $t_{0.5}$ ), 1 ( $t_1$ ), 3 ( $t_3$ ), 5 ( $t_5$ ), and 9 ( $t_9$ ) h of incubation.

The  $y$ -axis shows the relative absorbance. Peak 0, asialotransferrin; peak 1, monosialotransferrin; peak 2/3, di-trisialotransferrin; peak 4, tetrasialotransferrin; peak 5, pentasialotransferrin.

Storage of serum samples for 5 days did not interfere with the CDT measurement. The %CDT values were  $3.59\% \pm 0.33\%$  overall,  $3.59\% \pm 0.44\%$  after storage at  $4-8^\circ\text{C}$ ,  $3.76\% \pm 0.42\%$  after storage at room temperature, and  $3.68\% \pm 0.32\%$  after storage at  $37^\circ\text{C}$ . The between-day CV was 12% for samples stored at  $4-8^\circ\text{C}$ , 11% for samples stored at room temperature, and 8.8% for samples stored at  $37^\circ\text{C}$ .

#### INTERFERENCE TESTING

*Heterogeneity based on iron saturation.* The efficiency of on-column iron saturation was evaluated by comparing the analysis of 10 samples from patients with iron deficiency anemia with and without iron saturation before analysis. The differences in the percentages of monosialo- and di-trisialotransferrin were  $0.17\% \pm 0.32\%$  (mean peak area percentage,  $1.70\% \pm 0.57\%$ ) and  $0.06\% \pm 0.46\%$  (mean peak area percentage,  $3.53\% \pm 1.29\%$ ), respectively. The paired  $t$ -test revealed no significant difference in the percentages of the monosialo- and di-trisialotransferrin peak areas. No peaks migrating with the mobility of asialylated forms could be detected.

*Identification of transferrin variants and CDG.* To determine whether a healthy nonalcoholic carrier of a heterozygous CD variant of transferrin would give false-positive CDT values in our method, we compared the CZE pattern of CD variants with that of a healthy nonabuser and an alcohol abuser (Fig. 2). The transferrin CD variants showed a prominent tetrasialo-D peak with a migration time ( $9.89 \pm 0.03$  min) approximating that of the di-trisialylated C isoform. The heights of both the tetrasialo-C and -D peaks were very similar. A small peak with a migration time ( $9.75 \pm 0.06$  min) approximating that of the monosialo-C isoform could be designated as di-trisialo-D transferrin. The pentasialo-D peak migrated between the tetrasialo-D and tetrasialo-C peaks.

To investigate the resolution of the isoforms in CD variants, transferrin was gradually desialylated by increasing the incubation time with neuraminidase. Analyses (Fig. 3A) showed the subsequent appearance of the di-tri-, mono-, and asialylated D and C isoforms. In most of the D variants, small differences in migration times

**Table 1. Performance characteristics and analytical precision of the CZE method.<sup>a</sup>**

	Mono <sup>b</sup>	Di-Tri	Tetra
Migration time, min	$9.69 \pm 0.02$	$9.86 \pm 0.02$	$10.02 \pm 0.02$
CV for absolute migration time, %	0.15	0.15	0.16
CV for relative migration time, %	0.02	0.01	
Percentage of total peak area	$6.62 \pm 0.35$	$24.94 \pm 0.80$	$58.47 \pm 0.69$
CV, %	5.3	3.2	1.2
Theoretical plates	$312\ 000 \pm 21\ 000$	$199\ 000 \pm 6500$	$273\ 000 \pm 11\ 000$
Resolution		$2.22 \pm 0.05$	$1.87 \pm 0.03$
Asymmetry	$1.10 \pm 0.09$	$0.82 \pm 0.04$	

<sup>a</sup> Results are expressed as mean  $\pm$  SD; relative migration time CVs are calculated as ratios with the migration time of tetrasialotransferrin.

<sup>b</sup> Mono, monosialotransferrin; di-tri, di- plus trisialotransferrin; tetra, tetrasialotransferrin.



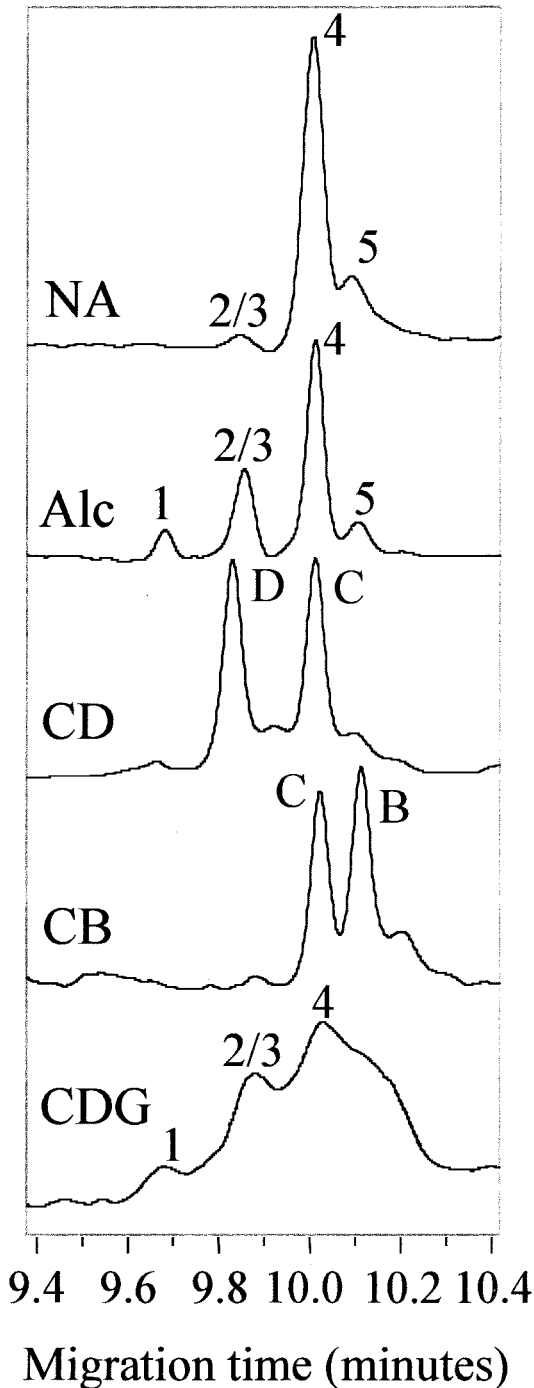


Fig. 2. Comparison of the transferrin spectra of samples from a healthy nonalcoholic (NA), an alcohol abuser (Alc), a heterozygous CD (CD) variant, and a CB variant (CB) of transferrin and a patient with CDG (CDG).

The y-axis shows the relative absorbance. Peak 1, monosialotransferrin; peak 2/3, di-trisialotransferrin; peak 4, tetrasialotransferrin; C, common transferrin phenotype; B, anodal B-transferrin variant; D, cathodal D-transferrin variant.

between the tetrasialo-D and the di-trisialo-C isotypes, the di-trisialo-D and the monosialo-C isotypes, and the monosialo-D and the asialo-C isotypes compromised individual

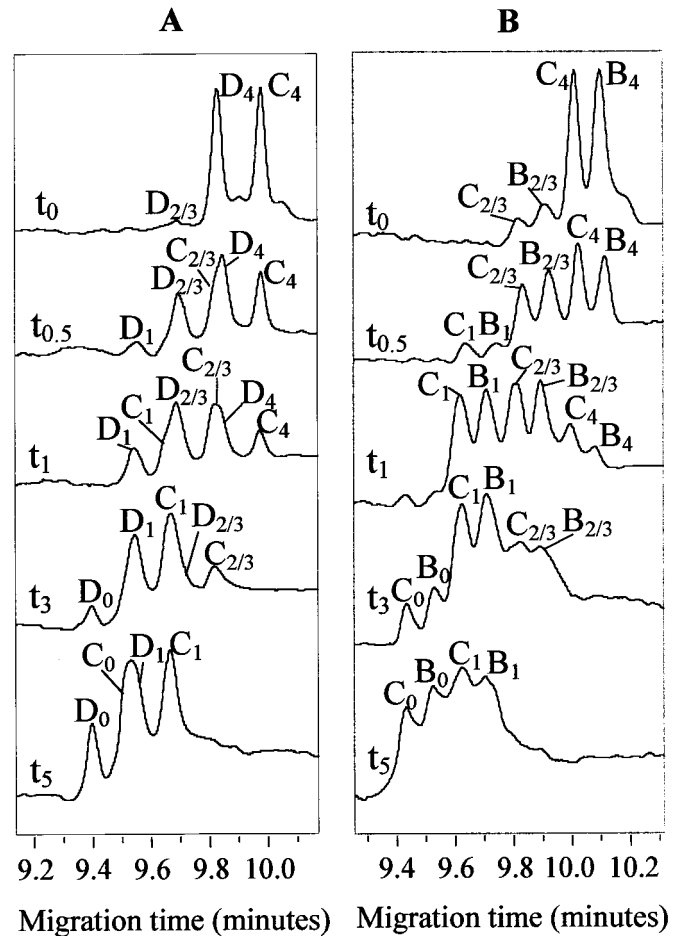


Fig. 3. Effect of neuraminidase treatment on the spectra of a heterozygous CD-transferrin variant (A) and a heterozygous CB-transferrin variant (B) without incubation ( $t_0$ ) and after 0.5 ( $t_{0.5}$ ), 1 ( $t_1$ ), 3 ( $t_3$ ), and 5 ( $t_5$ ) h of incubation.

The y-axis shows the relative absorbance. Peak  $C_0$ , asialo-C-transferrin; peak  $C_1$ , monosialo-C-transferrin; peak  $C_{2/3}$ , di-trisialo-C-transferrin; peak  $C_4$ , tetrasialo-C-transferrin; peak  $D_0$ , asialo-D-transferrin; peak  $D_1$ , monosialo-D-transferrin; peak  $D_{2/3}$ , di-trisialo-D-transferrin; peak  $D_4$ , tetrasialo-D-transferrin; peak  $B_0$ , asialo-B-transferrin; peak  $B_1$ , monosialo-B-transferrin; peak  $B_{2/3}$ , di-trisialo-B-transferrin; peak  $B_4$ , tetrasialo-B-transferrin phenotype.

integration of each transferrin peak. CDT integration of CD variants, including asialo-D, asialo-C, monosialo-D, monosialo-C, and di-trisialo-D, remained possible; however, comigration of the di-trisialo-C with the tetrasialo-D isoform excluded the di-trisialo-C peak from CDT.

To evaluate the influence of transferrin B isotypes on CDT analysis, we compared the CZE pattern of healthy nonalcoholic CB variants with that of a healthy nonabuser and an alcohol abuser (Fig. 2). The analysis of the CB variants demonstrated a prominent isoform with a migration time ( $10.12 \pm 0.06$  min) approximating that of the pentasialylated C isoform. The height of the tetrasialo-B peak was slightly higher than that of the tetrasialo-C peak because of comigration of the tetrasialo-B isotype and the penta-C-sialotransferrin. An additional peak migrating slower ( $10.20 \pm 0.05$  min) than the pentasialo-C isotype could be designated as the pentasialo-B transferrin (Fig.

2). Resolution of the isoforms in CB variants was investigated by analysis after neuraminidase treatment. These experiments (Fig. 3B) showed that the desialylated B- and C-transferrin peaks have discrepant migration times, doubling the number of transferrin peaks compared with the analysis of the serum of a healthy nonalcoholic volunteer after corresponding times of incubation with neuraminidase.

The electropherogram of the CDG patient revealed a minor tetrasialylated peak and increased di-trisialylated (29.0%) and monoglycosylated (5.4%) forms, leading to an increased CDT value of 34.4% (Fig. 2).

#### POPULATION DISTRIBUTION AND METHOD COMPARISON

The analysis of 130 sera from healthy nonalcoholic subjects demonstrated a gaussian distribution for the population according to the Kolmogorov–Smirnov test (Fig. 4). The 2.5th and 97.5th percentiles of the CDT values were 1.84% and 6.79% overall, 1.60% and 6.83% in the male population ( $n = 69$ ), and 2.32% and 6.70% in females ( $n = 61$ ). No significant gender or age difference was seen.

Capillary electrophoresis %CDT ( $y$ ) correlated with Axis %CDT TIA ( $x$ ) results in 77 serum samples of nonalcoholic, healthy subjects (social drinkers were included) and patients admitted to the Department of Psychiatry of the AZ Stuivenberg Hospital with suspicion of chronic alcoholism:  $y = 1.92x - 7.29$ ;  $r = 0.89$ ; Fig. 5. Deming regression revealed that in the low CDT range, CZE results were generally lower than the Axis %CDT-TIA values, and in the high range, CZE results were higher than the Axis %CDT-TIA values.

#### Discussion

Since its introduction, capillary electrophoresis has been proposed as an effective tool for clinical analysis (22–25). CDT determination by CZE has been demonstrated as reliable because of several advantageous characteristics of

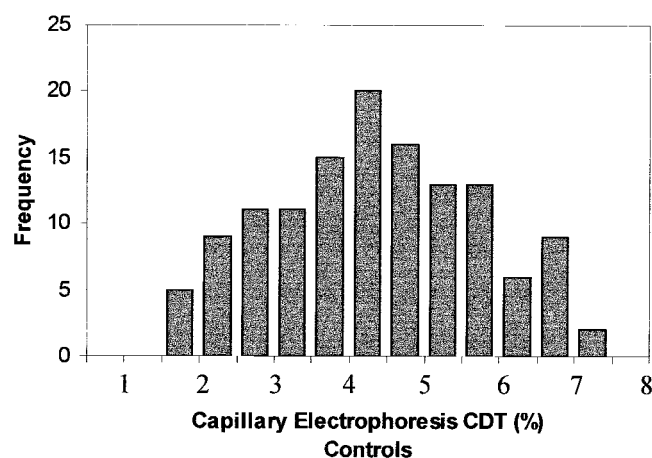


Fig. 4. Histogram of capillary electrophoresis %CDT values for 130 sera from healthy nonalcoholic subjects.

The distribution of the population was gaussian according to the Kolmogorov–Smirnov test.

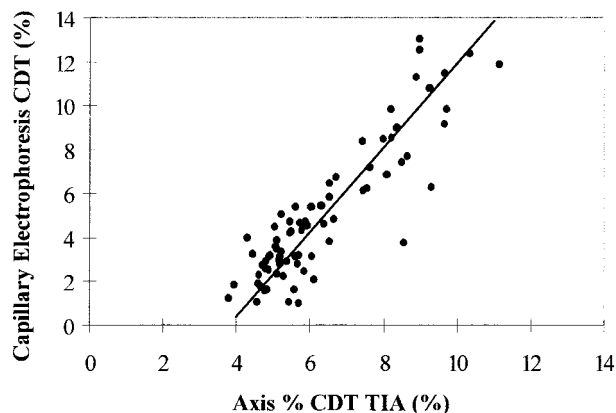


Fig. 5. Comparison of %CDT values obtained by the Axis %CDT TIA ( $x$ ) and capillary electrophoresis ( $y$ ) in 77 alcohol abusers and controls. The Deming regression equation was:  $y = 1.92x - 7.29$ ;  $r = 0.89$ .

capillary electrophoresis; nevertheless, there still remain areas for improvement. One of the problems in commonly used CDT methods is the relative imprecision as demonstrated by high interassay CVs for IEF (10–21%), HPLC (7.5–16%), fast protein liquid chromatography (7%), %CDT-TIA (10%) (26), CDTECT (10%) (27), and Chron AlcoI.D. (9%) (28). Tagliaro et al. (19) found relative peak area CVs of 4% for the disialo- and 9% for the trisialo-transferrins in their CZE method, showing the potential of CZE in improving precision. Our CZE method for CDT determination based on dynamic double coating revealed within-run CVs of 5.3% for the monosialo and 3.2% for the di-trisialo isoforms. Carbohydrate-deficient isoforms could be resolved on the basis of differences in migration times. Precision in CZE is strongly dependent on the stability of the electroosmotic flow, which can be monitored by the consistency of migration times. The within-run repeatability of the absolute and relative (calculated as ratios with the migration time of the tetrasialo isoform) migration times could be obtained because of the constant electroosmotic flow achieved by the combined effects of the high ionic strength of the buffer, the use of an extreme pH value, and dynamic double coating. The double layer of polymers, which is eliminated after each electrophoretic run and regenerated before the next, increases the number of negative charges on the capillary wall. These measures minimize protein adsorption to the capillary wall and maintain a constant electroosmotic flow from run to run and from capillary to capillary. The prevention of protein adsorption is necessary to obtain high numbers of theoretical plates and good peak symmetry. High voltages were applied to increase resolution because long migration times may cause peak broadening by diffusion. Between-day imprecision was relatively good (12%) compared with that in CDTECT (17%) (27), whereas ChronAlcoI.D. (28) revealed a comparable variability (11%). Nonoptimal storage had no significant influence on CDT determinations. In comparison, the improved CZE method (20) showed day-to-day CVs of 6.9%

for disialo- and 11% for trisialotransferrin in a pathological sample. Reliable electrophoretic separation of CDT might be complicated by the presence of the degradation product C3c of complement C3, which migrates at times equivalent to those for the asialo- and monosialotransferrins. The addition of anti-C3c antibodies in the running buffer eliminated the interfering degradation product to obtain a stable baseline.

Transferrin has two iron-binding sites per molecule, and under physiological circumstances serum transferrin is ~30% saturated with iron. Consequently, four different forms of transferrin with respect to iron content can be distinguished in human serum: apotransferrin, and N-terminal and C-terminal monoferric and diferric transferrin. The differences in iron saturation produce differences in charge. The electrophoretic migration of unsaturated transferrin overlaps the variation in sialic acid content. Interference of apo- or monoferric transferrin with CDT can be overcome by saturating the serum with iron (29, 30). Until now, the saturation has been performed before separation (precolumn mode) because it allows flexibility in optimizing reaction conditions and no complex instrumentation is needed. In the present CDT method, the iron is complexed to transferrin during the electrophoretic separation (on-column mode); the capillary column serves as the reactor vessel. Once injected onto the column, the sample is allowed to react with the iron present in the running buffer. In the on-column method, sample dilution is reduced compared with the precolumn complexation mode, making the on-column approach especially suitable for the analysis of biological samples that are already very dilute. Furthermore, introduction of a low-ionic strength sample into the iron-containing running buffer, which has a high ionic strength, induces sample stacking, increasing the resolution. In precolumn saturation, reducing the ionic strength of the sample by dilution can produce stacking; however, it requires long injection times for sufficient resolution (21). To obtain optimal complexation, the saturation should be fast and complete, no interfering side products should be formed, and the iron saturation should be stable. In vitro studies of iron release from transferrin revealed that acidification together with the reduction of Fe(II) ions to the Fe(III) state, at the cost of intracellular conversion of NADH to NAD<sup>+</sup>, are necessary in the release of iron from transferrin (30–32). The alkaline pH of the running buffer prevents the dissociation of iron from transferrin during electrophoresis. Our experiments on iron-deficient serum samples demonstrate sufficient transferrin iron-load stability.

Genetic variants of human serum transferrin were first discovered in 1957 by Smithies (33). In addition to the common (C) transferrin type, a series of anodal (B) and cathodal (D) variants have been reported in different human populations (7, 11, 34). The degree of genetic polymorphic variation in transferrin is limited in Caucasians. Most individuals express the C allele, and rarely,

heterozygous CB variants can be seen. In African Americans, the transferrin CD phenotype has a frequency of ~10% (35), and the highest frequencies of the D allele are seen in West Africa and western stream Bantu (36).

Our experiments demonstrated that CB and CD variants are easily detected by CZE analysis based on differences in migration times and the high similarity in peak heights between the C and B or D peaks. Neuraminidase treatment demonstrated that in CB variants, the respective carboxylated isoforms of the C and B isotypes migrated with different times so that peak integration of each isotype remained possible, but with loss of resolution. In CD variants, integration of the individual isotypes was compromised, and CDT was calculated as the sum of the di-trisialo-D, monosialo-C, monosialo-D, asialo-C, and asialo-D transferrins to avoid the risk of false-positive CDT values. Because the di-trisialo-C isoform is included in the non-CDT transferrin, reference values for D variants have to be established. IEF and HPLC allow detection of genetic variants, but methods using anion-exchange chromatography might give falsely increased CDT values in CD variants and miss the detection of variants because the eluted CDT fraction is quantified without visualization of the transferrin pattern (2, 4, 26). The coelution of tetrasialo-D with trisialo-C isoforms might cause strong overdetermination of CDT. Therefore, mainly anion-exchange chromatography-based tests that include trisialotransferrins are at risk for false-positive results (28). Recently, a comparison between CZE and immunoturbidimetry showed that the serum transferrin concentration in D variants might be underestimated when analyzed immunologically (37). Although transferrin concentrations in D variants were comparable to CC transferrin concentrations when measured by immunoturbidimetry, CZE areas under the curve differed significantly. We included the trisialylated isoform in addition to the asialylated, monosialylated, and disialylated isoforms in our CDT value because the metabolic process that converts high- to low-sialic acid transferrin is considered a continuous process and trisialotransferrin is increased in alcoholics (36). Furthermore, measurement of the different transferrin fractions separately could give additional information because disproportionate alcohol-induced increases in the different glycosylated isoforms might occur and additional information regarding the alcohol consumption over time could be provided. The clinical performance of separate fractions requires further evaluation.

In CDG patients, one-half of the transferrins lack two or all four of their terminal carbohydrate groups (38, 39). Like IEF and HPLC (2, 4, 26), CZE allows detection CDG. Our findings of markedly increased CDT in the serum of a CDG patient confirm previous observations (17). Recently, increased CDT values and abnormal protein glycosylation were demonstrated in a patient with achondroplasia, demonstrating the challenge in CDT determinations (40).

The comparison of CDT measurements by CZE and an

ion-exchange chromatographic method (Axis %CDT-TIA) revealed a significant correlation. Although the Axis %CDT-TIA method includes only 50% and our CZE method 100% of the trisialotransferrin, lower CZE values compared with the Axis results were seen in the lower CDT range. Comparable results have been described for IEF (41) and CDTECT (42). In spite of the high sensitivity of the Axis %CDT-TIA (41) method and the inclusion of only one-half of the trisialotransferrin, no %CDT values <3% could be obtained in our study or in previously reported data (41–43). The high correlation between the Axis %CDT-TIA and %CDT HPLC (41) was obtained because the %CDT-TIA minicolumn performance was calibrated so that the correlation between HPLC and TIA methods was at a maximum (44). This calibration produced an artificial increase in the Axis %CDT-TIA values in the lower region.

In conclusion, CZE is sufficiently sensitive to determine CDT isoforms with good precision and high resolution. The present method provides a CDT determination without any sample pretreatment, reducing manipulation errors and eliminating unnecessary dilution steps. Furthermore, we showed that CZE can easily detect genetic variants and CDG, reducing false-positive values, and that it is correlated with an ion-exchange chromatography-based method.

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