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Improved capillary electrophoresis method for the determination of carbohydrate-deficient transferrin in patient sera

Capillary zone electrophoresis (CZE) with a dynamic double coating formed by charged polymeric reagents represents an effective tool for the separation of iron-saturated transferrin (Tf) isoforms and thus the determination of carbohydrate-deficient transferrin (CDT, sum of asialo-, monosialo- and disialo-Tf in relation to total Tf) in human serum. Using the CEofix-CDT reagents, a 50 μm inner diameter (ID) capillary of 60 cm total length and the P/ACE MDQ under optimized instrumental conditions (20 kV and 30°C) is demonstrated to provide outstanding assay precision for the determination of CDT in human serum. For CDT levels of 1.0% and 4.5%, precision relative standard deviation (RSD) values ($n = 8$) were determined to be $< 3.0\%$ and $< 1.5\%$, respectively. During the first year of operation under routine conditions, more than 600 patient samples were analyzed in a total of 62 sets of runs. Except for selected samples of patients with severe liver diseases, interference-free Tf patterns were detected. Asialo-Tf was not detected in control sera and in patient sera with a CDT level $< 1.70\%$, but became detectable in 89.6% of sera with $> 2.3\%$ disialo-Tf. Monosialo-Tf was only detected in two sera containing $> 13.3\%$ CDT. The optimized CZE assay was applied to confirm positive CDT results produced by an immunoassay during long-term monitoring of a patient which led to the determination of the elimination kinetics of asialo-Tf, disialo-Tf, and CDT after an episode of high alcohol consumption (estimated apparent half lives of 4.86, 7.24, and 6.74 days, respectively). The optimized CZE assay with an upper reference limit for CDT of 1.70% represents an attractive alternative to high-performance liquid chromatography (HPLC). It features simpler sample preparation, faster analysis time, and higher isoform resolution compared to the most recent HPLC approach and can thus be regarded as a new candidate of a reference method for CDT.

Keywords: Asialotransferrin / Capillary zone electrophoresis / Disialotransferrin / Dynamic coating / Human serum / Monosialotransferrin
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1 Introduction

The abuse of alcohol represents one of the major public health problems in many different cultures with tremendous consequences for society, medicine, and economy. Self-reporting of alcohol consumption is notoriously unreliable and the diagnosis of alcohol abuse based on clinical symptoms is difficult. Therefore, laboratory markers are strongly required as diagnostic tools for an early and

more reliable detection of excessive alcohol consumption and alcoholism. A big variety of compounds have been investigated and some of them like γ -glutamyl transferase, aspartate aminotransferase, alanine aminotransferase, and mean corpuscular volume are widely used in the clinical practice, however, with questionable sensitivities and/or specificities [1–4].

Since its first description in the 1970s by Stibler *et al.* [5–7], carbohydrate-deficient transferrin (CDT) has gained more and more attention and importance in the detection of chronic, heavy alcohol consumption and in the monitoring of alcohol abusers, including the detection of relapse drinking. CDT encompasses isoforms of the glycoprotein transferrin (Tf) with zero up to two sialic acid residues in the carbohydrate side chains of the molecule. Tf, the most important iron-transporting protein, contains two carbohydrate chains with a total of up to eight

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Abbreviations: CDT, carbohydrate-deficient transferrin; CRP, C-reactive protein; NCCLS, National Committee for Clinical Laboratory Standards; Tf, transferrin; TIA, turbidimetric immunoassay

end-standing sialic acid residues, with the major isoform – accounting for about 80% of total Tf – being tetra-sialo-Tf with four sialic acid residues [2, 8]. Today, CDT is considered as the most specific biological marker for the identification of chronic excessive alcohol intake [8, 9].

In the last two decades, different analytical approaches for the quantitative determination of CDT in human serum have been developed, including isoelectric focusing, anion-exchange chromatography followed by immunochemical determinations, high-performance liquid chromatography (HPLC), and capillary zone electrophoresis (CZE) [1, 2, 4, 10–12]. With the adoption of dynamic capillary coatings, preventing proteins from adsorbing to the capillary surface, CZE in fused-silica capillaries turned out to be an effective analytical approach for the separation and quantitative determination of the Tf isoforms and thus CDT [2, 13–16]. Recently, commercial reagents producing a dynamic double coating and serving as the separation buffer (CEofix-CDT kit; Analis, Namur, Belgium) have been introduced and shown to provide the best performance characteristics in terms of reproducibility, detector response, and analysis time [16]. To produce the bilayer of the double coating, the fused-silica capillary is first rinsed with an initiator solution containing a polymeric polycation (such as Polybrene) that is adsorbed to the wall surface followed by introduction of the running buffer with a polymeric polyanion (such as poly(vinylsulfonate)) that is forming a second layer and thus providing the negative charge for a strong electroosmotic flow towards the cathode. Further investigations with this CDT assay revealed outstanding precision performance according to the internationally accepted National Committee for Clinical Laboratory Standards (NCCLS) EP5-A protocol [17] as well as high clinical sensitivity and specificity that were determined by receiver operating characteristic (ROC) plots [18, 19]. The bottleneck of the CEofix-CDT test was identified as being the incomplete separation between di- and trisialo-Tf [16, 17], a problem that was recently addressed and optimized *via* reduction of applied voltage and temperature. In the same work, different peak integration schemes were evaluated and the health-associated reference limits of CDT were determined under different instrumental conditions [20].

In this paper, we wish to report the assessment of the precision performance of the optimized CEofix-CDT-based method for the quantification of CDT and to provide insight into the first experience with this method under routine conditions for screening and confirmation analysis of CDT in patient sera. A discussion on the occurrence of asialo- and monosialo-Tf and the long-term monitoring of a patient revealing an episode of relapse drinking are also presented.

2 Materials and methods

2.1 Reagents and samples

Buffers and reagents of the CEofix-CDT kit for the quantification of CDT in human serum with the P/ACE MDQ (Kit No. 10-004740) were obtained from Analis (Namur, Belgium). Rabbit anti-human Tf antibody (titer: 2800 mg/L) was from Dako (Glostrup, Denmark) and 0.9% sodium chloride solution from Bichsel (Interlaken, Switzerland). Patient sera were from the departmental analytical laboratory where they were received for determination of CDT. They were stored in 8.0 mL polypropylene tubes at -20°C . Control sera were divided into 60 μL aliquots and stored in 0.5 mL polypropylene vials at -20°C .

2.2 Sample preparation

Frozen samples were defrosted in a water bath at 37°C for 15 min and vortex-mixed for a few seconds. Then, 60 μL of sample and 60 μL of the ferric solution of the reagent kit (Analis) were combined in a 0.5 mL polypropylene vial and vortex-mixed. No further incubation time for iron saturation of the sample was required, and the vial was put into the corresponding sample tray of the P/ACE MDQ (Beckman-Coulter, Fullerton, CA, USA). For immunosubtraction of Tf, 80 μL of serum was incubated with 160 μL of anti-human Tf antibody in a polypropylene vial for 45 min at room temperature. After centrifugation at $8000 \times g$ and 4°C for 20 min, 60 μL of the supernatant was collected and combined with 30 μL of the ferric solution. For data comparison with untreated sample, 30 μL of serum was combined with 60 μL of 0.9% NaCl solution and 45 μL of the ferric solution.

2.3 Instrumentation, data evaluation, and running conditions

A P/ACE MDQ capillary electrophoresis system (Beckman-Coulter), equipped with a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm ID (375 μm OD) and 60.2 cm (50 cm to the detector) total length, was used. Samples were introduced from 0.5 mL polypropylene vials by applying a negative pressure of 0.3 psi (1 psi = 6894.76 Pa) for 9–10 s. The temperature control of the circulating cooling fluid of the capillary cartridge was set to 30.0°C and the temperature of the sample tray was kept at 15.0°C . The applied voltage was 20.0 kV, resulting in a current of about 14.4 μA and a power level of 0.48 W/m. It is important to note that these instrumental conditions are different from those proposed by the manufacturer of the CEofix-CDT kit that were employed in a previous publication [17] and are those

found to provide optimized resolution between disialo- and trisialo-Tf without changing capillary dimensions and running buffer [20]. UV detection was effected with a 200 nm interference filter. The 32 Karat software, Version 4.01 (Beckman) was used for data registration and evaluation. Peak areas were determined by valley-to-valley integration and the data were evaluated on the basis of corrected peak areas (peak area divided by detection time). The amounts of single Tf isoforms and CDT (sum of asialo-, monosialo-, and disialo-Tf) were calculated as area % in relation to the sum of the corrected peak areas of all detected Tf isoforms. New capillaries were rinsed for 5 min with 0.2 M NaOH with a pressure of 20 psi from the inlet end to the outlet end, followed by 0.2 M NaOH by simultaneous application of a pressure of 20 psi from the inlet end and a constant current of 80 μ A for 10 min. The same procedure was employed for the conditioning at the beginning of a new day. At the end of a day, the capillary was rinsed from the inlet to the outlet side with 0.2 M NaOH and water for 5 min each with a pressure of 60 psi, and the capillaries were stored wet with the ends kept in water. Between runs the capillary was first rinsed with 0.2 M NaOH for 1 min with a pressure of 60 psi applied at the inlet end, followed by 0.2 M NaOH by applying simultaneously a pressure of 20 psi from the inlet to the outlet end and a constant current of 80 μ A for 1.5 min. At the beginning of each run, the capillary was first conditioned from the inlet side with the initiator buffer by applying a pressure of 15 psi for 1.5 min and then with the run buffer for 2 min by applying the same pressure. All Tf isoforms were detected within 12 min and the total time interval required for analysis and conditioning was 22 min.

2.4 CDT determination with the %CDT turbidimetric immunoassay test and determination of serum alcohol

CDT was determined with the turbidimetric immunoassay %CDT TIA of Axis-Shield (distributed through Bio-Rad, Hercules, CA, USA) according to the recommendations of the kit manufacturer using the Cobas Mira plus (Roche Diagnostics, Rotkreuz, Switzerland). A CDT value < 2.6% of total Tf was considered to be within the normal range. Serum alcohol was determined on the Vitros 250 using the Vitros ALC slide technology (Ortho Clinical Diagnostics, Neckargemünd, Germany).

3 Results and discussion

3.1 Precision performance and quality control

It was shown in a previous publication that the CEofix-CDT kit, employed according to the instructions of the manufacturer, resulted in an excellent precision perfor-

mance. The bottleneck of the assay, however, was identified to be the incomplete separation of disialo- and trisialo-Tf, with the resolution between these two peaks being typically < 1.3 for sera of healthy individuals and alcohol abusers [17]. Without changing the capillary dimensions and the composition of the running buffer, baseline separation of di- and trisialo-Tf could be achieved by lowering the applied voltage and the temperature of the capillary cartridge [20], resulting in the optimized method described in this manuscript.

The precision performance of the optimized method in regard to migration times of Tf isoforms and quantification of single Tf isoforms and CDT was evaluated. Customary intraday and interday precision data were calculated for a serum of a healthy volunteer containing a CDT amount below the cut-off value, referred to as “sample low”, and for a patient serum with an elevated CDT level, referred to as “sample high”. Electropherograms of the two sera are depicted in Fig. 1. For the intraday data, eight samples of

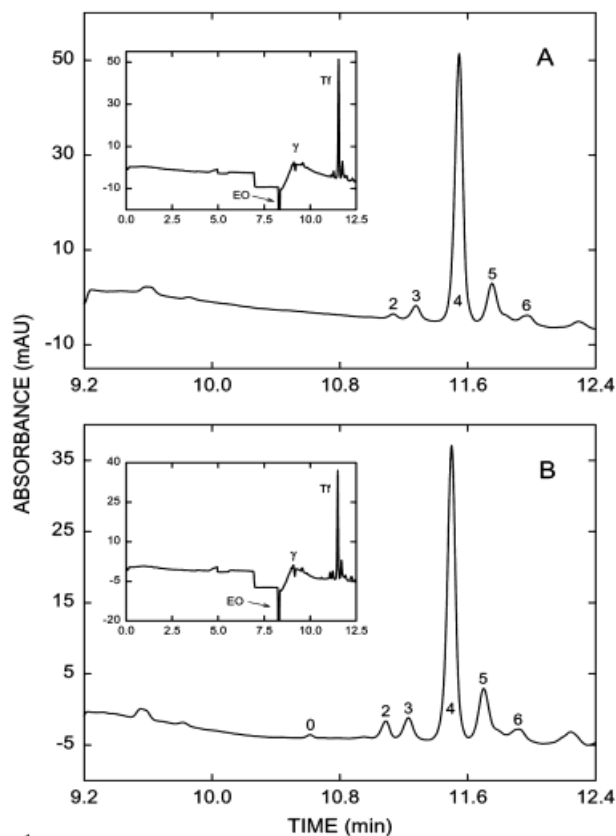


Figure 1. Typical CZE Tf patterns obtained with a sample containing (A) a normal CDT level of 1.05% and (B) an elevated CDT level of 4.6%. The inserts depict the entire electropherograms. EO and γ refer to electroosmosis peak and γ -region, respectively. Isoform levels are given in Table 1 and experimental conditions are provided in Sections 2.2 and 2.3. Key: 0, asialo-Tf; 2, disialo-Tf; 3, trisialo-Tf; 4, tetrasialo-Tf; 5, pentasialo-Tf; 6, hexasialo-Tf.

each serum were prepared separately and analyzed in one series. For the gathering of the interday data, one sample of each serum was prepared and analyzed on eight consecutive working days. The precision data are presented in Tables 1 and 2. RSD values for the intraday precision of migration times of all Tf peaks were found to range between 0.18 and 0.29%. Corresponding values

for the interday data were between 0.58% and 0.66%, values that are more than twofold lower compared to those obtained employing the instrumental conditions proposed by the manufacturer referred to as the standard method [17]. In agreement with previous findings [17], RSD values for the quantitative determination of single Tf isoforms and CDT were found to be dependent on the iso-

Table 1. Intraday precision data for single transferrin isoforms and CDT ($n = 8$)

Tf isoform(s)	Detection time			Isoform or CDT amount		
	Mean (min)	Confidence interval (min)	RSD (%)	Mean (%)	Confidence interval (%)	RSD (%)
Sample low						
Disialo-Tf	11.160	11.143–11.177	0.18	1.057	1.032–1.081	2.78
Trisialo-Tf	11.302	11.284–11.319	0.19	4.321	4.284–4.357	1.01
Tetrasialo-Tf	11.575	11.556–11.593	0.20	78.418	78.372–78.464	0.07
Pentasialo-Tf	11.780	11.760–11.800	0.20	13.482	13.443–13.521	0.35
Hexasialo-Tf	12.002	11.981–12.023	0.21	2.722	2.678–2.767	1.94
CDT (asialo- + disialo-Tf)	–	–	–	1.057	1.032–1.081	2.78
Sample high						
Asialo-Tf	10.636	10.616–10.657	0.23	0.699	0.675–0.723	4.16
Disialo-Tf	11.114	11.091–11.138	0.25	3.862	3.818–3.906	1.36
Trisialo-Tf	11.254	11.230–11.277	0.25	5.577	5.559–5.595	0.38
Tetrasialo-Tf	11.527	11.502–11.552	0.26	72.270	72.169–72.370	0.17
Pentasialo-Tf	11.728	11.702–11.754	0.27	14.729	14.684–14.773	0.36
Hexasialo-Tf	11.945	11.916–11.973	0.29	2.864	2.828–2.900	1.51
CDT (asialo- + disialo-Tf)	–	–	–	4.561	4.517–4.605	1.15

Table 2. Interday precision data for single transferrin isoforms and CDT ($n = 8$)

Tf isoform(s)	Detection time			Isoform or CDT amount		
	Mean (min)	Confidence interval (min)	RSD (%)	Mean (%)	Confidence interval (%)	RSD (%)
Sample low						
Disialo-Tf	11.045	10.991–11.098	0.58	1.054	1.036–1.072	2.07
Trisialo-Tf	11.184	11.129–11.239	0.59	4.319	4.278–4.360	1.14
Tetrasialo-Tf	11.450	11.393–11.507	0.59	78.608	78.461–78.754	0.22
Pentasialo-Tf	11.654	11.596–11.711	0.59	13.418	13.329–13.507	0.79
Hexasialo-Tf	11.873	11.814–11.933	0.60	2.602	2.534–2.670	3.11
CDT (asialo- + disialo-Tf)	–	–	–	1.054	1.036–1.072	2.07
Sample high						
Asialo-Tf	10.554	10.501–10.607	0.60	0.680	0.661–0.700	3.43
Disialo-Tf	11.025	10.967–11.083	0.63	3.904	3.857–3.951	1.44
Trisialo-Tf	11.164	11.105–11.223	0.63	5.574	5.541–5.606	0.70
Tetrasialo-Tf	11.432	11.371–11.493	0.64	72.529	72.372–72.685	0.26
Pentasialo-Tf	11.631	11.568–11.694	0.65	14.581	14.467–14.695	0.94
Hexasialo-Tf	11.852	11.786–11.917	0.66	2.733	2.649–2.816	3.64
CDT (asialo- + disialo-Tf)	–	–	–	4.584	4.540–4.628	1.15

form level, with the lowest RSD values (< 0.26%) for tetrasialo-Tf and the highest for asialo-Tf (< 4.2%) (Tables 1 and 2). The observed RSD values are 2–3.5 times lower than those obtained with the standard method [17, 20].

The quantification and detection limits for single isoforms were assessed for asialo- and disialo-Tf and determined to be 0.10% and 0.05%, respectively. Peaks leading to values lower than 0.10% were not accounted for the determination of CDT. For the “sample low” with a CDT level of about 1.06%, no asialo- and monosialo-Tf could be detected. Intraday and interday RSD values for CDT were found to be 2.78% and 2.07%, respectively. No monosialo-Tf could be detected in the “sample high” whose CDT level was determined to be about 4.6% and intraday and interday RSD values were found to be 1.15% (Tables 1 and 2).

For internal quality control, a control sample stemming from the serum of a healthy volunteer was included in each set of runs. The monitored electropherogram did not exhibit an asialo-Tf peak. In the first year of routine use of the assay, 62 sets of samples were analyzed. The same capillary was employed during the entire time period during which a total of about 1000 analyses (total of patient, control, and research samples) were analyzed and there were no instrumental problems. The mean (RSD) disialo-Tf or CDT level calculated from the 62 values was determined to be 1.01% (3.79%). Similarly, the mean (RSD) trisialo-Tf amount in the same sample was found to be 4.19% (2.24%). These data document both the excellent precision of the assay and the above discussed dependence of isoform level on the RSD value. Thus, the data generated thus far demonstrate that the optimized method is very robust and reproducible.

3.2 Determination of CDT in patient samples

As discussed in detail elsewhere [20], the CDT reference limits were calculated by nonparametric methods from the 2.5th and 97.5th percentiles of the disialo-Tf reference values of 54 reference samples (asialo-Tf was not detected in these samples) that stemmed from individuals with no or moderate (≤ 20 g ethanol per day) alcohol consumption. For the optimized separation conditions, the mean of the CDT values was 1.13% (SD: 0.18%; median: 1.11%; range: 0.80–1.59%) and the upper (90% confidence interval) and lower (90% confidence interval) reference limits were determined to be 1.57% (1.50–1.63%) and 0.81% (0.74–0.88%), respectively. Based on these data, the threshold value used for routine analysis was set to 1.7% which is essentially the mean +3SD.

The optimized CZE assay was introduced as routine method for the determination of CDT levels in patient sera in our departmental analytical laboratory. During the first year of operation, more than 600 samples were analyzed. The majority of samples provided straightforward patterns such as those depicted in Fig. 1. Sera whose electropherograms showed unusual peak distributions were reanalyzed after immunosubtraction of Tf (Fig. 2) and/or identified as discussed previously [17] (Fig. 3). A total of six sera revealed heterozygous Tf-BC variants (Fig. 3A) and one serum showed a heterozygous Tf-CD variant. Five samples comprised elevated peaks in the γ -globulin region (Fig. 3B), and specimens with elevated C-reactive protein (CRP) levels were monitored with a peak detected shortly before disialo-Tf

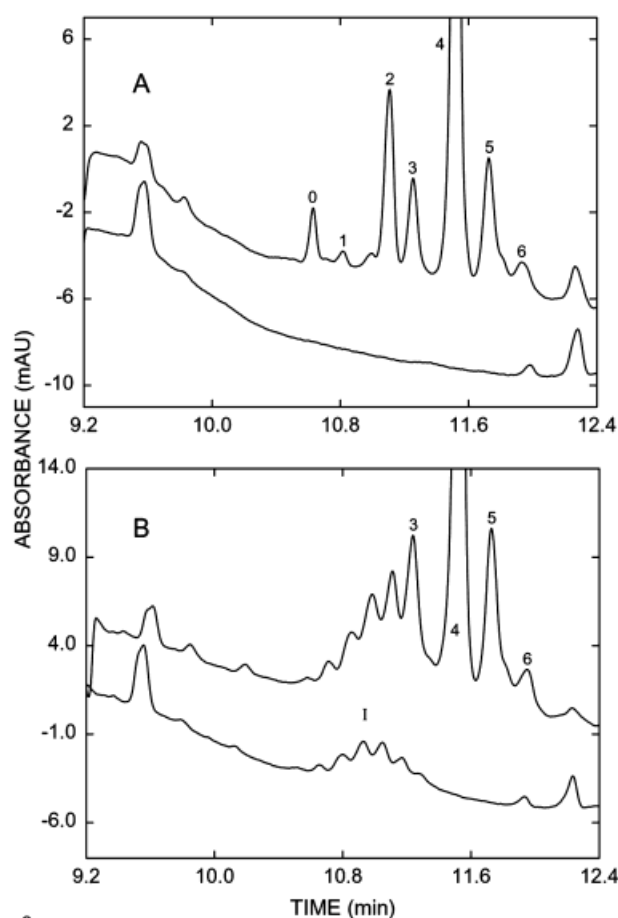


Figure 2. Electropherograms before (upper graphs) and after (lower graphs) immunosubtraction of Tf obtained with (A) the serum of an alcohol abuser that revealed the presence of monosialo-Tf and (B) the serum of a patient comprising an unknown, multiple-peak interference (marked with I in lower graph). The CDT level of the serum whose data are depicted in (A) was determined to be 18.77% (14.28% of disialo-Tf, 3.59% of asialo-Tf, and 0.90% of monosialo-Tf). For experimental conditions refer to Sections 2.2 and 2.3. Key as in Fig. 1.

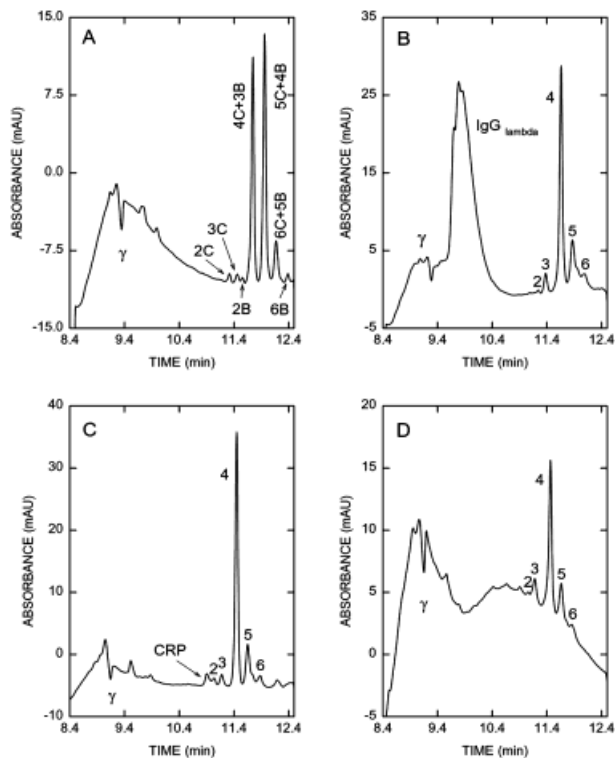


Figure 3. CZE electropherograms obtained for (A) a heterozygous Tf-BC variant, (B) a sample with a large peak in the γ -region (IgG_{lambda}, same sample as for Fig. 6A in [17]), (C) a serum with an elevated CRP level of 93 mg/L, and (D) a serum with a broad interference in the Tf region. For experimental conditions refer to Sections 2.2 and 2.3. Key as in Fig. 1.

(Fig. 3C). Furthermore, for selected samples stemming from patients treated at the hepatology outpatient clinic, a broad interference under the Tf pattern was detected (Fig. 3D). Elevated immunoglobulin A levels are assumed to be the reason for the interference. However, our efforts in removing the interfering compounds failed thus far and more work is required for elimination and identification of this problem. Two sera of one patient drawn 6 months apart revealed the presence of an unknown interference that prevented the determination of CDT with this assay (Fig. 2B).

The levels of asialo- and disialo-Tf of part of the 589 patient samples that could be unambiguously evaluated are depicted in Fig. 4 (presented in the order of increasing disialo-Tf values) and the frequency diagrams of disialo-Tf and CDT levels of all 589 samples are shown in Fig. 5. Disialo-Tf levels ranged between 0.31% and 14.41% (mean: 1.52%, median: 1.18%), asialo-Tf was detected in 71 samples whereas asialo-Tf was quantitated in 49 sera (range: 0.14%–3.63%, mean: 0.85%, median: 0.41%). CDT levels were found to be between 0.31% and 18.04% (mean: 1.59%, median: 1.18%). In serum

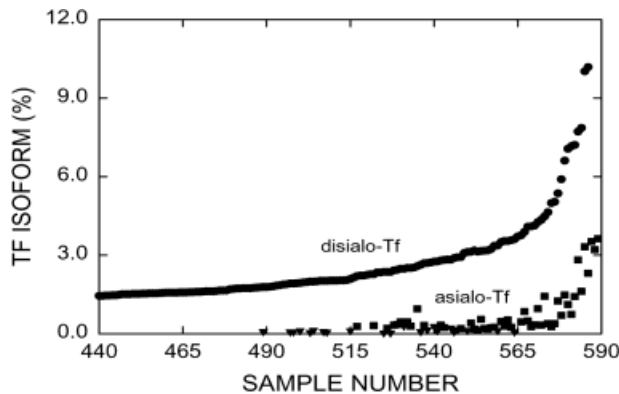


Figure 4. Disialo-Tf (circles) and asialo-Tf (triangles for detectable peaks between 0.05 and 0.1% and squares for quantitated peaks $\geq 0.1\%$) levels of the samples 440 to 589 presented in the order of increasing disialo-Tf values.

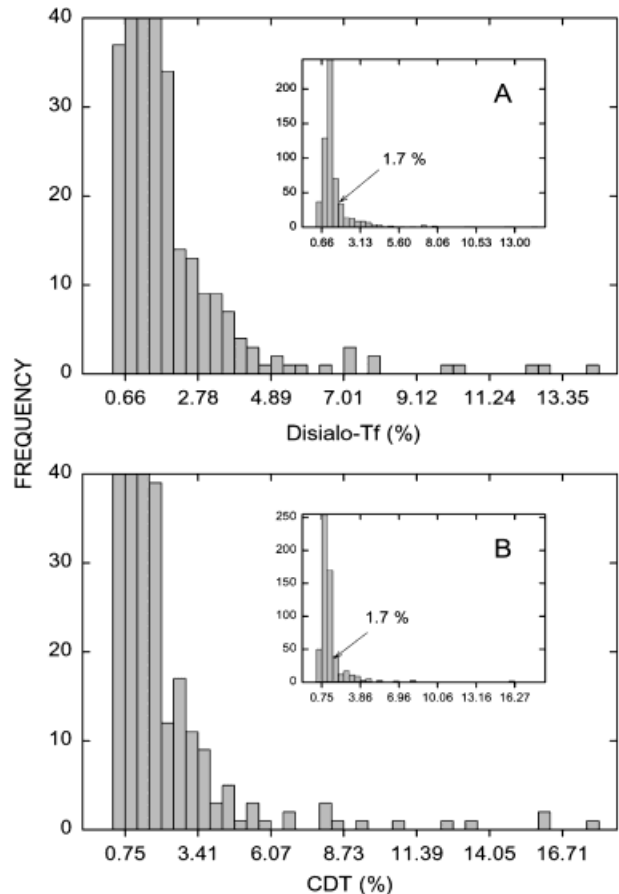


Figure 5. Frequency diagrams of (A) disialo-Tf and (B) CDT levels of the 589 patient samples. The widths of the bars are 0.35% and 0.44%, respectively.

samples revealing CDT levels below the upper reference limit determined for this assay (1.70%, total of 479 samples), no asialo-Tf was detected. The same was essen-

tially found to be true for samples with disialo-Tf levels below 2.30% (up to sample 522). Up to this level, small asialo-Tf peaks could be monitored in 11 (quantitated in 2) sera only (Fig. 4). Asialo-Tf was detected in 60 (quantitated in 47) of the 67 samples with disialo-Tf levels > 2.30%. The fact that in some cases even in sera with CDT levels up to 3.71% no asialo-Tf was present may be explained by the inter-individual variation of the response in CDT isoform formation [21, 22].

In a patient sample with a CDT value of 18.77% (14.28% of disialo-Tf, 3.59% of asialo-Tf), monosialo-Tf was detected with a relative amount of 0.90% (Fig. 2A, upper graph). Based on the data obtained after immunosubtraction (Fig. 2A, lower graph), and from the pattern change obtained after neuraminidase treatment [17], the peak detected after 10.81 min (relative migration time compared to tetrasialo-Tf of 0.939) was assigned to monosialo-Tf. Mårtensson *et al.* [23] reported monosialo-Tf to be present in trace amounts in the sera of social drinkers. These monosialo-Tf concentrations are believed to be below the detection limit of our CZE assay. Furthermore, the monosialo-Tf increase during chronic alcohol consumption is reported to be much less pronounced compared to the increase of the other CDT isoforms [23]. These data are in good agreement with our findings as no peak could be attributed to monosialo-Tf in samples with CDT levels < 13.3%. It can be speculated that monosialo-Tf reaches the detection limit of the employed

CZE assay only after chronic intake of highest amounts of alcohol, producing extremely high values of asialo- and disialo-Tf.

3.3 Long-term CDT monitoring

Although the value of CDT for identifying high-risk drinkers and alcohol abusers is still discussed controversially, with reported test sensitivities and specificities depending on the investigated patient collective and control group, the study design and the analytical principle employed, CDT is widely used for assisting the diagnosis of alcohol abuse and alcohol dependence [2, 3, 24–26]. Longitudinal CDT monitoring of individuals with a history of alcohol abuse is important for the early detection of relapse drinking after abstinence and in legal cases [4, 25]. The use of an individual cutoff drawn from an abstinent or drinking baseline of the monitored person, instead of a common upper reference limit derived from the statistical distribution of a reference sample group (see above), has been recommended for that purpose. The biological variation of CDT values between individuals can thereby be overcome, while the intra-individual serum CDT levels are reported to be fairly constant in teetotalers and social drinkers [2, 21, 22, 25, 27–29].

CDT of one patient was monitored with the turbidimetric immunoassay %CDT TIA of Axis-Shield over an extended period of time (for values refer to the last column in Table 3).

Table 3. Levels of Tf isoforms and CDT of a patient over a period of 134 days

Time (day)	Capillary electrophoresis							%CDT TIA
	Asialo-Tf (%)	Disialo-Tf (%)	Tri-sialo-Tf (%)	Tetra-sialo-Tf (%)	Penta-sialo-Tf (%)	Hexa-sialo-Tf (%)	CDT (%)	CDT (%)
1	ND	0.98	7.26	75.15	13.54	3.07	0.98	2.23
10	ND	0.96	7.18	75.00	13.74	3.11	0.96	1.74
31	2.68	8.86	6.40	65.92	13.54	2.59	11.54	9.39
36	1.12	4.94	8.46	69.73	12.93	2.82	6.06	6.35
43	0.46	2.97	8.29	72.74	12.86	2.68	3.43	4.29
50	0.17	1.90	7.49	74.90	12.97	2.58	2.07	3.29
57	ND	1.64	6.96	75.03	13.70	2.67	1.64	2.52
64	ND	1.37	7.07	75.24	13.82	2.50	1.37	2.34
71	ND	1.20	7.29	75.58	13.60	2.33	1.20	2.14
78	ND	1.02	7.07	75.77	14.07	2.07	1.02	1.92
86	ND	0.97	7.43	75.30	13.34	2.96	0.97	1.94
93	ND	0.94	6.50	76.69	13.79	2.08	0.94	1.79
100	ND	1.03	7.38	75.79	13.43	2.37	1.03	1.95
106	ND	0.95	6.59	76.38	13.63	2.44	0.95	1.81
113	ND	0.97	6.73	76.28	13.65	2.37	0.97	1.89
120	ND	0.98	6.62	76.77	13.40	2.24	0.98	1.75
127	ND	0.93	6.28	76.71	13.79	2.30	0.93	1.78
134	ND	1.00	6.26	76.68	13.52	2.53	1.00	1.80

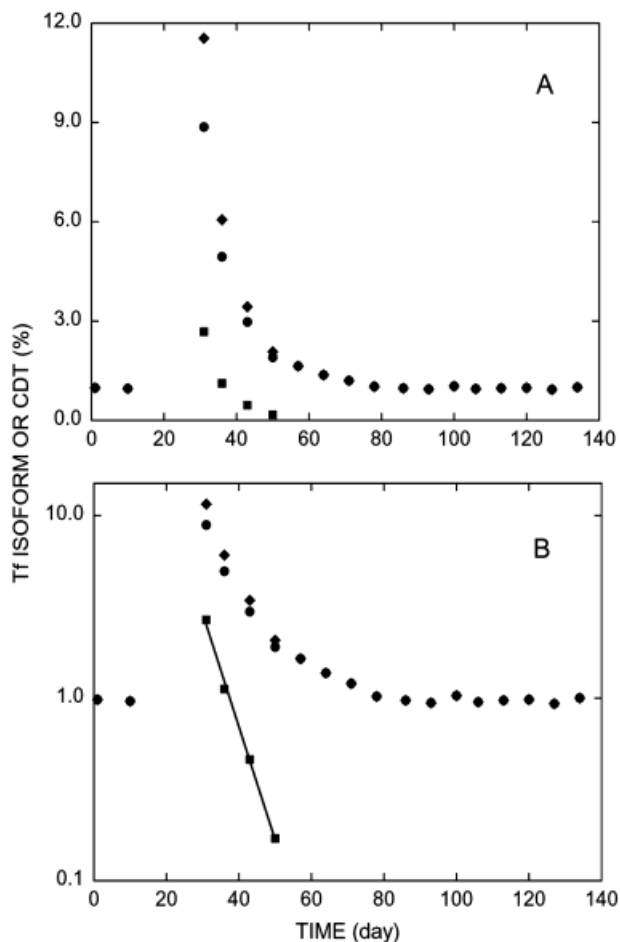


Figure 6. CZE data of long-term CDT monitoring: Disialo-Tf (circle), asialo-Tf (square) and CDT (diamond) levels of a patient determined by CZE during a 134-day period. The data in (B) are the same as those of (A) but presented in a semi-log format. The straight line in (B) represents the regression line for asialo-Tf ($y = -0.0619x + 2.3196$, $r = 0.9984$).

Although alcohol could not be determined in these samples, CDT was found to be markedly elevated at days 31, 36, 43, and 50, with CDT values being above the upper reference limit of 2.6% adopted for this immunoassay according to the manufacturer's instructions and above the 3.0% threshold value often employed in clinical practice [30]. As this increase was unexpected, the samples were subjected to confirmation analysis by CZE. The time courses of the thereby determined levels of single CDT isoforms and CDT are presented in Fig. 6 and the values of all Tf isoforms and CDT are summarized in Table 3. Figure 7 depicts selected electropherograms. Using CZE, the four samples in question were also found to be positive. Linear regression analysis of the two sets of data revealed a linear relationship with $\text{CDT}(\% \text{CDT TIA}) = 0.744 \times \text{CDT}(\text{CZE}) + 1.256$ ($r = 0.990$, $n = 18$).

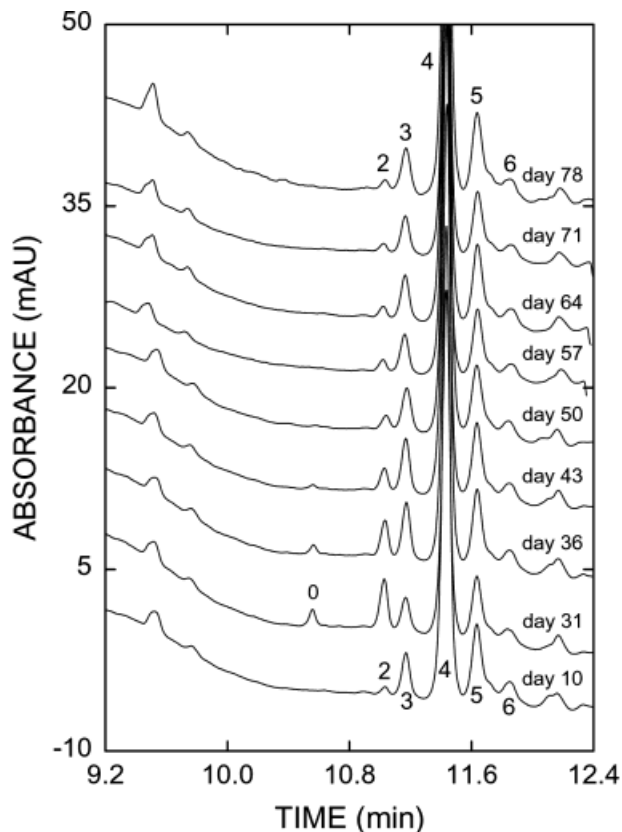


Figure 7. Selected electropherograms for the data of Fig. 6. The data are presented with y-axis offsets and the x-axis being adjusted for equal appearance of tetrasialo-Tf. For experimental conditions refer to Sections 2.2 and 2.3. Key as in Fig. 1.

With the CZE-based confirmation analyses data, detailed information about the temporal behavior of all Tf isoforms is obtained. The first two samples, drawn within ten days, showed almost identical CDT values (0.98% and 0.96%) and no asialo-Tf (Table 3). These CDT values are similar to the mean of the reference samples (see above), are far below the upper reference limit of the method (1.70%) and are considered to represent the baseline value of this patient (see below). Three weeks later, about a twelvefold increase of CDT (11.54%) with detectable asialo-Tf (2.68%) was monitored. Thereafter, a continuous decrease of the levels of asialo-Tf, disialo-Tf and thus CDT was noticed during the following 7 weeks, reaching the previous baseline value of CDT at day 78 (Fig. 6A). CDT levels above the cutoff value of 1.70% were obtained up to day 50. Asialo-Tf was detected in the same time interval and monosialo-Tf could not be detected at any time. From days 57–71, CDT was below the upper reference limit of the assay (1.70%) but above the individual patient's baseline value. The level of asialo-Tf was found to decrease exponentially (Fig. 6B). Using a

PC-compatible, one-compartment model for elimination of an exogenous compound (Excel 97 SR2, PK functions for Microsoft Excel, www.boomer.org), the apparent half life of the elimination was determined to be 4.86 days. Disialo-Tf and CDT serum levels did not decrease in the same manner (Fig. 6B) and apparent half lives were estimated to be 7.24 and 6.74 days, respectively. These values compare well with those reported in the literature [1–4, 31].

The mean value of the 11 baseline values (samples at days 1, 10, and 78 to 134) was calculated to be 0.98% \pm 0.03%. The considerably smaller variation compared to that reported by Borg *et al.* [28] can at least partly be attributed to the much higher reproducibility of the CZE assay (see Section 3.1.) compared to that of the immunoassay for which an assay precision of 10% is reported. Adopting the method described in [28] for identification of relapse drinking, namely an increase of CDT from the minimum or baseline value of three or four times the coefficient of variation of the baseline values, would lead to a cutoff level for this patient of 1.07% to 1.10%. These findings are in good agreement with the data of Anton *et al.* [22] who reported a mean elevation of CDT levels of 10% among relapse drinkers during a twelve-week outpatient trial.

Trisialo-Tf values varied between 6.26% and 8.46% (mean: 7.07%, RSD: 8.77%). For the 11 samples with baseline CDT values, mean and RSD were determined to be 6.85% and 6.28%, respectively. The highest trisialo-Tf level was detected for the sample at day 36 with its value being 23.5% above the mean value of the samples with baseline CDT. Whether this small increase can be attributed to an effect related to the alcohol intake is unclear. In agreement with the literature, no significant change in the trisialo-Tf levels could otherwise be observed [8, 32, 33]. Not surprisingly, compared to disialo-Tf, tetrasialo-Tf, and to a much smaller extent pentasialo-Tf showed a reciprocal change of the relative amount. The lowest value of tetrasialo-Tf (13.3% below the 76.09% mean value of the 11 samples with baseline CDT values) was noticed when CDT was highest (day 31), while the lowest value of pentasialo-Tf (5.6% less than the 13.63% mean value of samples revealing baseline values of CDT) was determined with a time shift of twelve days (day 43). Hexasialo-Tf did not change significantly during the entire episode.

There is still some confusion about the daily amount of ethanol and the duration of regular alcohol intake needed to elevate CDT. Reports claiming that daily intake of 50–80 g pure ethanol over a period of at least one week resulted in increased CDT levels [1–4, 7, 34] are contradicted by drinking experiments with healthy individuals, concluding that an intake of up to 60–80 g ethanol per

day for three weeks did not increase CDT [35] or showed a significant but only moderate elevation without reaching the upper reference limit for most individuals [36]. It was speculated that the response of CDT to alcohol consumption of subjects with no drinking history may be different from that of individuals with alcohol dependence, leading to sensitization caused by regular drinking [3, 22, 35, 37, 38]. The huge increase of CDT found in the monitored patient was unexpected and can only be a result of very high alcohol consumption between days 10 and 31.

4 Concluding remarks

Using the CEofix-CDT reagents and the P/ACE MDQ under optimized instrumental conditions (20 kV and 30°C) is demonstrated to provide outstanding assay precision for the determination of CDT in human serum. Compared to the conditions recommended by the kit manufacturer (28 kV and 40°C), resolution between disialo- and trisialo-Tf is improved and complete such that the CDT-relevant Tf isoforms can be quantitated precisely. Intraday and interday precision data for asialo- and disialo-Tf were found to be isoform level dependent. For isoform levels of about 0.7%, 1.0%, and 4.0%, RSD values ($n = 8$) were determined to be < 4.2%, < 3.0%, and < 1.5%, respectively. During the first year of operation under routine conditions, more than 600 patient samples were analyzed in a total of 62 sets of runs and without changing the capillary. The control sample analyzed in this one-year period ($n = 62$) revealed RSD values of 3.79% and 2.24% for mean isoform levels of 1.01% and 4.19%, respectively. Except for selected samples received from patients being treated at the hepatology outpatient clinic, interference-free Tf patterns were detected. Asialo-Tf was not detected in control sera and in patient sera with a CDT level < 1.70%, but became detectable in 89.6% of sera with > 2.3% disialo-Tf. Monosialo-Tf was only detected in a few sera containing > 13.3% CDT. The optimized CZE assay was applied to confirm positive CDT results produced by an immunoassay during long-term monitoring of a patient which led to the determination of the elimination kinetics of asialo-Tf, disialo-Tf, and CDT after an episode of high alcohol consumption. Apparent elimination half lives could thereby be estimated to be 4.86, 7.24, and 6.74 days, respectively. The optimized CZE assay with an upper reference limit for CDT of 1.70% represents an attractive alternative to HPLC. It features simpler sample preparation, faster analysis time, higher isoform resolution and much simpler column reconditioning compared to the most recent improved HPLC approach [12]. The CZE approach can thus be employed for both, screening and confirmation

analysis alike and fulfills the requirements of a reference method for CDT. The improved assay could also be used for legal cases in forensics.

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