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Capillary zone electrophoresis with a dynamic double coating for analysis of carbohydrate-deficient transferrin in human serum Precision performance and pattern recognition

Christian Lanz[a], Ulrich Marti[b], Wolfgang Thormann[a,*]

[a]*Department of Clinical Pharmacology, University of Berne, Murtenstrasse 35, CH-3010 Berne, Switzerland*
[b]*Department of Clinical Chemistry, Inselspital, CH-3010 Berne, Switzerland*

Abstract

Capillary zone electrophoresis (CZE) with a dynamic double coating permits the simultaneous, individual, quantitative determination of transferrin (Tf) isoforms in human serum and thus carbohydrate-deficient transferrin (CDT), the most specific marker available today for the detection of chronic, excessive alcohol intake. CZE of serum Tf was carefully evaluated using the P/ACE MDQ with fused-silica capillaries of 50 μm I.D. and 60.2 cm total length, the CEofix CDT kit and the instrumental conditions recommended by the kit manufacturer. The precision performance assessed over a 20-day period according to the internationally accepted NCCLS EP5-A guidelines revealed the CZE assay as being highly reproducible with within-run and total precision being dependent on the Tf isoform level and RSD values ranging between 2.2 and 17.6%. Inter-day RSD values for asialo-Tf were noted to be between 9.8 and 11.5% and for disialo-Tf between 3.8 and 8.6%, whereas those for CDT levels of 0.87 and 4.31% of total Tf were determined to be 8.6 and 3.4%, respectively. The RSD values for trisialo-Tf, tetrasialo-Tf, pentasialo-Tf and hexasialo-Tf were found to be between 0.4 and 4.1%. Tf patterns are recognized and identified via detection times of Tf isoforms (intra-day and inter-day RSD values $<1.0\%$ and $<1.7\%$, respectively), immunosubtraction of Tf and enzymatic sequential cleavage of sialic acid residues. Furthermore, heterozygous Tf BC and Tf CD variants are assigned via spiking with a known mixture of Tf isoforms (e.g. the serum of a healthy Tf C homozygote). Among the non-Tf peaks monitored, the CRP peak detected shortly before disialo-Tf was identified by immunosubtraction and peak magnitudes were found to correlate well with immunochemically determined CRP serum levels. The CZE assay with dynamic double coating could thereby be shown to be sensitive enough to determine elevated CRP levels in human serum. Furthermore, unusual peaks in the γ-region were identified by customary serum protein CZE, immunosubtraction CZE and immunofixation.

Keywords: Pattern recognition; Precision; Coated capillaries; Transferrins; Proteins

1. Introduction

In the 1970s, Stibler et al. observed an increase of transferrin (Tf) isoforms with isoelectric points ≥5.7 in the serum and cerebrospinal fluid from patients with a chronic or repeated excessive alcohol intake [1–3]. Tf, the most important iron transporting protein, consists of 679 amino acids forming two iron binding sites and bears two N-linked carbohydrate chains that are composed of neutral sugars and of zero up to eight end-standing, negatively charged

*Corresponding author. Tel.: +41-31-632-3288; fax: +41-31-632-4997.
E-mail address: wolfgang.thormann@ikp.unibe.ch (W. Thormann).

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sialic acid residues. The major Tf isoform, accounting for about 80% of whole Tf in human serum, contains four sialic acid residues and is known as tetrasialo-Tf with an isoelectric point of 5.4 [4,5]. Isoforms with p*I* values ≥5.7 encompass Tf molecules with two or less sialic acid residues and are therefore collectively named carbohydrate-deficient Tf (CDT). CDT is widely accepted as the most specific marker available today for the detection of chronic, excessive alcohol intake [6]. Different analytical approaches—based on isoelectric focusing, immunochemical determination of fractionated Tf isoforms and high-performance liquid chromatography (HPLC)—have been employed for the investigation of Tf isoforms and the determination of CDT. Many of the developed methods are either time-consuming or, due to possible interferences, prone to analytical inaccuracy [7,8]. During the last decade, capillary zone electrophoresis (CZE) has been extensively applied to the determination of Tf isoforms and thus also CDT. Among the various methods developed, CZE in dynamically coated fused-silica capillaries proved to be the most promising approach [8–16].

Dynamic capillary coatings are obtained with buffer additives that are being adsorbed onto the capillary via formation of a dynamic equilibrium. Macromolecule interactions with the capillary wall can thereby be prevented or at least minimized and the capillary is newly resurfaced for each analysis [17]. For the determination of CDT in human serum, our group recently compared three CZE methods with different dynamic capillary coatings, employing diaminobutane, spermine and a commercial double coating [9]. During that work, the double coating approach was identified as being the best. In this approach, the fused-silica capillary is first rinsed with an initiator buffer containing a polymeric polycation that is adsorbed to the wall surface followed by introduction of the running buffer containing a polymeric polyanion that is forming a second layer and thus providing the negative charge for a strong electroosmotic flow (EOF) towards the cathode. The CEofix CDT-kit of Analis (Namur, Belgium) comprises proprietary ionic polymers [18] that form a bilayer by noncovalent adsorption, such as the bilayers produced by Polybrene/dextran sulfate [19], poly(diallyldimethylammonium)/poly-(styrene sulfonate) [20] and Polybrene/poly-(vinylsulfonate) [21]. The EOF in these systems was determined to be essentially independent of pH and highly reproducible.

CZE of Tf in human serum allows the quantitative determination of CDT [8,9,16]. Moreover, it also provides information about the whole Tf pattern and other anionic serum compounds that are migrating slower than Tf and are thus detected ahead of Tf. Abnormalities in the Tf pattern [13,14] and the γ-region [15] can thereby be visualized also. There are currently major efforts being undertaken with the aim of a widespread adoption of the CZE assays for CDT into the routine arena, both for screening and confirmation in clinical and forensic analysis [8,9,14–16]. During the past year, the performance of the newly introduced commercial CZE kit for CDT (CEofix CDT-kit of Analis) on the P/ACE MDQ capillary electrophoresis system of Beckman Coulter (Fullerton, CA, USA) was carefully evaluated using the instrumental conditions recommended by the kit manufacturer. We now wish to report (i) the precision performance data that were established according to internationally accepted NCCLS EP5-A guidelines [22], (ii) investigations of the nature of different abnormalities in the Tf pattern and the γ-region, and (iii) the identification of the C-reactive protein (CRP) peak present in many sera in varying amounts and detected directly in front of disialo-Tf.

2. Experimental

2.1. Chemicals

If not stated otherwise, chemicals were of analytical grade. Rabbit anti-human Tf antibody (titer: 2800 mg/l) and rabbit anti-human C-reactive protein antibody (titer: 900 mg/l) were purchased from Dako (Glostrup, Denmark). Neuraminidase type X from *Clostridium perfringens* (209 units/mg solid, 240 units/mg protein) and neuraminidase type III from *Vibrio cholerae* (3 mg protein/ml, 3 units/mg protein) were obtained from Sigma (St. Louis, MO, USA). *N*-Glycosidase F (100 units) was purchased from Roche Diagnostics (Rotkreuz, Switzerland). Buffers and reagents of the Analis CEofix-CDT-kit for quantitation of CDT with the P/ACE MDQ (kit

no. 10-004740) were kindly provided by Analis. It is important to note that this kit comprises a buffer similar but not identical to that employed by Wuyts et al. [14]. It is, however, believed to be the same as that now furnished for the P/ACE 5500 recently employed by Legros et al. [16].

2.2. Serum samples

Patient sera were obtained from the departmental analytical laboratory where they were received for determination of CDT. Our own sera were used as sera of healthy persons. All blood samples, drawn by venipuncture, were collected in native plastic tubes without additives (Monovette, white cap, Sarstedt, Sevelen, Switzerland). After clotting, the blood samples were centrifuged at room temperature for 10 min at 2000 *g*. The supernatant was transferred into 8.0-ml polypropylene tubes. All sera were stored at −20 °C until use.

2.3. Sample preparation

Sera were slowly defrosted and vortex mixed at room temperature. According to the manufacturer's instructions, 60 μl of serum and 60 μl of the ferric solution of the kit (Analis) were mixed directly in the microtiter plate (Beckman Coulter). The sample was mixed gently by aspiration and release of part of the fluid with a plastic pipette. No incubation time was required for iron saturation of the serum. For identification of Tf isoforms in the presence of genetic Tf variants, 30 μl serum were combined with 30 μl of a control serum of a healthy person prior to the addition of the 60 μl of ferric solution.

2.4. Instrumentation, running conditions and data evaluation

All CZE measurements and data evaluation were performed according to the instructions of the manufacturer of the CEofix CDT-kit (Analis). Briefly, a P/ACE MDQ capillary electrophoresis system (Beckman Coulter) that was equipped with a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 60.2 cm (50.0 cm to the detector)×50 μm I.D. was employed. The O.D. of the capillary was about 360 μm. For the evaluation of the precision performance, capillaries with the same length and identical inner and outer diameter as described above were received from Analis. New capillaries were rinsed for 10 min with 0.2 *M* NaOH with a pressure of 40 p.s.i. applied from the inlet end (1 p.s.i.=6894.76 Pa). The same procedure was employed at the beginning of each day. At the end of a day the capillary was washed with 0.2 *M* NaOH and water (5 min each with 60 p.s.i. from the inlet to the outlet end). The capillary was stored wet and the ends were kept in water. Between runs the capillary was rinsed for 1 min with 0.2 *M* NaOH and a pressure of 60 p.s.i. from the inlet end and then with 0.2 *M* NaOH for 1.5 min by applying simultaneously a pressure of 20 p.s.i. at the inlet end and a constant current of 80 μA. At the beginning of a new run, the capillary was conditioned by applying a pressure of 15 p.s.i. from the inlet to the outlet side with the initiator buffer for 1.5 min followed by the run buffer of the CDT kit for 2 min. The iron saturated sample was introduced from the microtiter plate by applying a vacuum of 0.3 p.s.i. for 10 s at the outlet end. The temperature controls of the cartridge and sample tray were set to 40 and 15 °C, respectively. The voltage applied was 28 kV (anode at the inlet side) resulting in a current of about 26 μA and a power level of about 1.21 W/m. UV detection was effected by using a 200 nm interference filter. Data registration, evaluation and storage were carried out with the 32 Karat Software, version 4.01 (Beckman). Data evaluation was based upon corrected peak areas of single isoforms (peak areas determined by valley-to-valley integration and divided by detection time) and are presented as area% in relation to the sum of the peak areas of all detected Tf isoforms. In contrast to the previous work from our laboratory [9], the amount of CDT was not evaluated as Tf index (area% of disialo-Tf in relation to tetrasialo-Tf) but as area% of the sum of asialo-Tf and disialo-Tf in relation to the sum of the peak areas of all Tf isoforms.

2.5. Evaluation of precision performance

The precision performance of the Analis CEofix-CDT-kit was evaluated according to the EP5-A protocol from NCCLS [22]. A serum with a low level of CDT and one with a high amount of CDT,

referred to as ''sample low'' and ''sample high'', respectively, and two corresponding controls referred to as ''control low'' and ''control high'', were analyzed over a period of 20 working days. The ''sample low'' was prepared from about 8 ml serum of a healthy volunteer. The serum was homogenized and a total of 120 60-μl aliquots were stored in 0.5-ml polypropylene vials at −20 °C until use. The ''control low'' stemmed from another healthy person and was prepared in the same way. The ''sample high'' was prepared from 16 patient sera that were combined, vortex mixed, divided into 90 aliquots of 60 μl each, and stored at −20 °C until use. The ''control high'' was a serum pool of four patient samples and was stored as 45 aliquots of 60 μl each.

The evaluation was performed by one operator and started after the operator was familiar with the handling of all processes. Two separate sets of runs were analyzed on each day and the time period between the end of the first and beginning of the second set was at least 2 h. Each sample in each set was analyzed in duplicate, resulting in a total of eight measurements per set or a total of 16 runs per day. For each set, the two aliquots of the ''sample low'', ''sample high'' and ''control low'' were prepared separately. For the ''control high'', one aliquot was prepared only and sample injection occurred twice from the same vial. The order of analysis was determined randomly for each set and the initiator and run buffers were changed every 100 analyses. All kits and reagents had the same lot number and capillaries from one lot were employed. Electropherograms were evaluated by valley-to-valley integration and the values of asialo-Tf and disialo-Tf were reported for all samples.

The values of the controls were reported on control charts including the mean values with two, three (warning limit) and four (out-of-control limit) standard deviations (SD). Every 5 days the mean value and the corresponding SD were recalculated for each chart from all acceptable data collected thus far. At day 5, an additional set of runs was performed comprising 20 separately prepared aliquots of the ''sample low''. From these data a preliminary within-run precision was calculated according to the procedure stated in the protocol. All unexpected events and problems and their solutions were reported in detail. After collection of the data of 20 acceptable operating days, the within-run precision and the total precision of the CZE method for the determination of CDT was calculated according to the statistics described in detail in the protocol. In addition, relative standard deviations (RSDs) for within-run and total precision were calculated by dividing the SD by the corresponding mean value of the 80 measurements and multiplication with 100.

2.6. Immunosubtraction of Tf and CRP

For immunosubtraction of Tf, 100 to 400 μl of serum were incubated in a plastic vial with varying relative volumes of anti-human Tf antibody. The smallest relative amount of anti-human Tf antibody was a quarter of the volume of the investigated serum, and the largest relative volume of antibody corresponded to four times the volume of serum. The sample was incubated at room temperature for 15 min to 2 h. Then, the incubate was centrifuged at 8000 *g* for 20 min at 4 °C and the supernatant was collected immediately and measured without further preparation. For data comparison with untreated sample, serum was equally diluted with water after iron saturation and analyzed under the same conditions. For immunosubtraction of CRP, 60–200 μl of serum were vortex mixed in a plastic vial with a volume of anti-human CRP antibody corresponding to 1% of the volume of the serum up to a volume of antibody that was twice the volume of the investigated serum. After incubation at room temperature for 0.5–2 h, the sample was centrifuged at 8000 *g* for 20 min at 4 °C. The supernatant was removed immediately and analyzed after saturation with an equal volume of the ferric solution of the CDT-kit (Analis). For some experiments the incubation of serum with anti-human CRP antibody was performed in the presence of the ferric solution. For investigation of the antibody solution, the reagent was diluted in water (2–20-fold) prior to analysis.

For sequential immunosubtraction of CRP and Tf in the same serum sample, 190 or 180 μl of serum were incubated in a plastic vial with 10 or 20 μl, respectively, of anti-human CRP antibody at room temperature for 1 h. Then, the precipitate was removed via centrifugation at 8000 *g* for 20 min at 4 °C. Fifty μl of the collected supernatant were saturated with 50 μl of the ferric solution of the kit

(Analis) as described in Section 2.3 and analyzed. The Tf immunosubtraction was performed with 60 μl of the supernatant via addition of 120 μl of the anti-human Tf antibody. The sample was incubated for 1 h at room temperature. After centrifugation for 20 min at 8000 *g* and 4 °C, the supernatant was collected immediately and analyzed without further preparation.

2.7. Digestion with neuraminidase and N-glycosidase F

For digestion with neuraminidase type X, the enzyme was reconstituted to 1 U/ml with 50 m*M* sodium acetate (pH 5.0, adjusted with acetic acid). Sixty μl of serum were saturated in a plastic vial with 60 μl of the ferric solution of the CEofix CDT-kit and combined with 60 μl of the enzyme solution. After vortex mixing, the sample was first incubated at 15 °C for 20.5 h with the microtiter plate sitting in the garage of the CE instrument, then at 30 °C for 2 h and then at 37 °C for 6 h. This sample was analyzed at various time points between 0 and 28.5 h. Prior to the measurements at 26 h and 28.5 h, 90 μl and 50 μl, respectively, of water were added to the sample. For digestion with neuraminidase type III, 360 μl of serum were combined with 3 μl of the purchased enzyme solution. After vortex mixing, the sample was incubated at 37 °C for 120 h. For each analysis, a 30-μl aliquot was withdrawn from the sample and combined with 30 μl of the ferric solution of the CEofix CDT-kit. For digestion with *N*-glycosidase F, 50 μl of serum were combined with 10 μl of the reconstituted solution (100 U/100 μl water). After vortex mixing, the sample was first incubated at 37 °C for 3.5 h. Then, 40 μl of the ferric solution were added and aliquots were analyzed at various time intervals during the following 24-h incubation at 37 °C.

2.8. Routine assays for CRP, serum proteins and CDT

CRP was determined in the routine laboratory with the CRP Tina-quant kit (Roche Diagnostics) on a Hitachi 917 Analyzer (Roche Diagnostics). CZE serum protein analyses and immunosubtraction CZE analyses were performed according to the manufacturer's instructions using the Paragon CZE 2000 Clinical Capillary Electrophoresis System (Beckman, Brea, CA, USA) and the provided reagents (Beckman). Immunofixation analysis of serum proteins on agarose gels was performed with Hydrasis (Sebia, Issy-les-Moulineux, France). For other serum protein patterns obtained with these techniques refer to Ref. [23]. CDT of older sera were determined with the %CDT turbidimetric immunoassay (Bio-Rad, Hercules, CA, USA) whose CDT values <6.0% were considered to be within the normal range. Newer sera were monitored with the %CDT TIA turbidimetric immunoassay of Axis-Shield (Bio-Rad), an assay with a CDT cut-off value of 2.6%.

3. Results and discussion

3.1. Precision performance

Analytical methods have to be investigated carefully before use in a routine arena and their capability of generating useful results has to be evaluated. They are fully described by their reliability criteria encompassing specificity, accuracy, precision and detectability, and their practicability criteria summarizing information about speed, cost, technical skill requirements and dependability. These criteria describe the performance characteristics of an analytical method [24]. In this manuscript, the precision performance, as an important part of the reliability of a method, of the commercially available CDT-kit is reported. It has been evaluated according to the NCCLS EP5-A guideline, an internationally accepted protocol that provides guidance and procedures for the evaluation of the precision of in vitro diagnostic devices. The variability of a device when used over a long period of time is of highest interest for the description of the precision performance. Several time-related components contribute to this total precision and can be characterized as within-run, between-run, within-day and day-to-day precision. The NCCLS document is designed to estimate the total precision and within-run precision [22]. Typical electropherograms for all four samples analyzed are presented in Fig. 1 and Table 1 summarizes the performance data obtained in our study. Standard deviations and the corresponding values of the

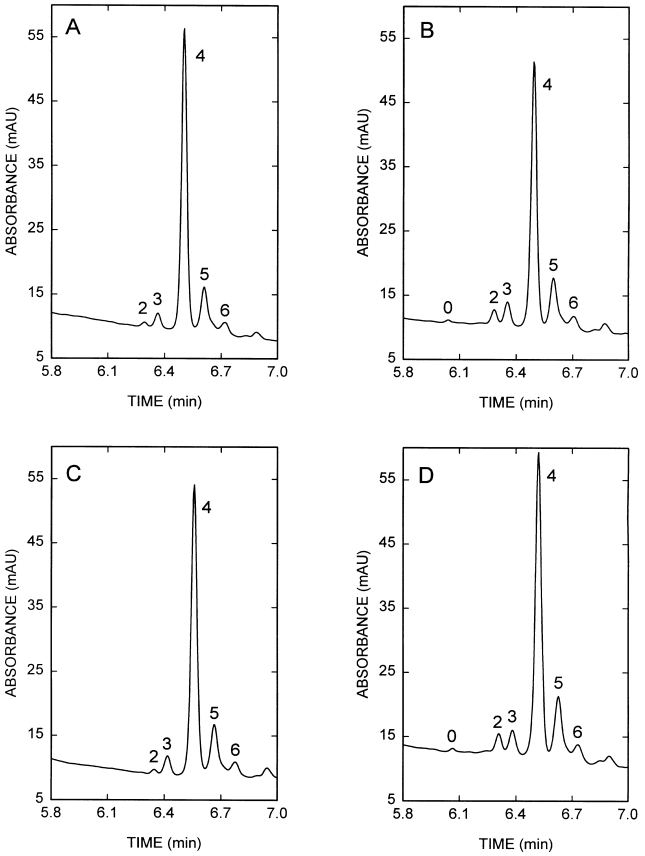

Fig. 1. Typical CZE Tf patterns obtained with (A) ''sample low'', (B) ''sample high'', (C) ''control low'' and (D) ''control high''. Mean area% values of the Tf isoforms are listed in Tables 1 and 3. Key: 0, asialo-Tf; 2, disialo-Tf; 3, trisialo-Tf; 4, tetrasialo-Tf; 5, pentasialo-Tf; 6, hexasialo-Tf.

Table 1
Within-run and total precision data according to the NCCLS EP5-A protocol

Sample	Isoform	Isoform level[a] (area%)	Within-run precision			Total precision		
			SD (area%)	Degrees of freedom[b]	RSD (%)	SD (area%)	Degrees of freedom[c]	RSD (%)
Sample low	Disialo-Tf	0.88	0.044	40	5.03	0.064	61	7.28
Sample high	Asialo-Tf	0.68	0.098	40	14.39	0.120	66	17.64
	Disialo-Tf	3.20	0.090	40	2.80	0.124	57	3.87
Control low	Disialo-Tf	0.93	0.040	40	4.26	0.062	53	6.72
Control high	Asialo-Tf	0.75	0.065	40	8.68	0.068	63	9.08
	Disialo-Tf	3.59	0.079	40	2.19	0.128	54	3.57

[a] Mean of all 80 measurements performed during the 20-day evaluation. Data expressed in area% of total Tf.
[b] Number of series (degrees of freedom for SD).
[c] Degrees of freedom for total standard deviation estimate (rounded to nearest integer).

degrees of freedom were calculated according to the protocol. Additionally, the mean of all data collected over the 20-day period ($n=80$) was determined and used to calculate RSD values. Except for asialo-Tf of the ''sample high'', RSD values are shown to be below 10%. For the within-run precision and the total precision, RSD values were determined to be as low as 2.19% and 3.57%, respectively. For both, RSD values are becoming smaller as the isoform level is increasing. The detection limit for asialo-Tf and any other Tf isoform was estimated to be about 0.05% of total Tf, whereas the limit of quantification was taken as 0.10% of total Tf.

The precision performance evaluation according to the NCCLS EP5-A guideline is based upon duplicate analyses that are performed twice a day. Routine analyses are typically run once only which prompted us to evaluate the customary intra-day and inter-day precision data as well. The intra-day data correspond to the preliminary within-run precision data obtained with the ''sample low'' on day 5 (for an electropherogram refer to Fig. 1A) and are summarized in the top line of Table 2. Corresponding data for the other Tf isoforms and the detection times for all isoforms are also included in Table 2. RSD values for detection times and isoform quantitation were found to be smaller than 1.0% and 5.1%, respectively, values that correspond well to the preliminary data published previously [9]. Similar data were obtained for a sample with detectable asialo-Tf that was assessed outside the NCCLS EP5-A protocol (lower part of Table 2). Furthermore, the inter-day data presented in Table 3 are those obtained after evaluation of the results of the first aliquots of the four samples analyzed each day during the 20-day period. Again, RSD values for isoform quantitation were found to be dependent on peak size. For asialo-Tf, RSD was $<12\%$ whereas for all other isoforms $<8.6\%$. RSD values of detection times were determined to be $<1.7\%$ in all cases (Table 4). For the quantitation of asialo-Tf and disialo-Tf, the values presented in Tables 2 and 3 were found to correspond well with those of Table 1. RSD values for CDT were determined to be between 5.07% and 8.55% for samples containing normal amounts of CDT. Corresponding values for samples with elevated CDT levels were between 1 and 4% (Tables 2 and 3). These data indicate that the CE method is highly reproducible and precise.

During the execution of the precision performance

Table 2
Intra-day precision data ($n=20$)

Tf isoform(s)	Detection time			Isoform or CDT amount		
	Mean (min)	SD (min)	RSD (%)	Mean[a] (area%)	SD (area%)	RSD (%)
Sample low[b]						
Disialo-Tf	6.329	0.057	0.90	0.914	0.046	5.07
Trisialo-Tf	6.399	0.058	0.91	3.848	0.047	1.23
Tetrasialo-Tf	6.538	0.061	0.94	79.930	0.281	0.35
Pentasialo-Tf	6.644	0.064	0.96	12.603	0.200	1.59
Hexasialo-Tf	6.752	0.066	0.98	2.706	0.096	3.53
CDT (asialo-Tf + disialo-Tf)	–	–	–	0.914	0.046	5.07
Sample high[c]						
Asialo-Tf	6.175	0.023	0.38	0.442	0.030	6.85
Disialo-Tf	6.439	0.027	0.41	3.650	0.041	1.13
Trisialo-Tf	6.516	0.028	0.43	4.250	0.146	3.43
Tetrasialo-Tf	6.667	0.030	0.44	75.938	0.247	0.32
Pentasialo-Tf	6.781	0.031	0.46	13.164	0.100	0.76
Hexasialo-Tf	6.897	0.033	0.48	2.557	0.038	1.47
CDT (asialo-Tf + disialo-Tf)	–	–	–	4.091	0.044	1.07

[a] Isoform or CDT amount expressed in area% of total Tf.
[b] Data assessed at day 5 of the precision evaluation and thus represents the preliminary within-run precision of the EP5A protocol.
[c] Different serum pool as employed for the precision evaluation (Table 1) and inter-day precision data (Tables 3 and 4).

Table 3
Inter-day precision data for quantitation of single transferrin isoforms and CDT ($n=20$)[a]

Tf isoform(s)	Sample low		Sample high		Control low		Control high	
	Mean (area%)	RSD (%)	Mean (area%)	RSD (%)	Mean (area%)	RSD (%)	Mean (area%)	RSD (%)
Asialo-Tf	ND	ND	0.679	11.54	ND	ND	0.761	9.82
Disialo-Tf	0.873	8.55	3.173	4.41	0.923	6.65	3.550	3.81
Trisialo-Tf	3.769	3.13	5.863	2.13	4.912	1.50	5.178	2.37
Tetrasialo-Tf	80.219	0.44	72.786	0.58	77.320	0.40	72.361	0.62
Pentasialo-Tf	12.524	1.76	14.303	1.01	13.836	1.74	14.639	1.62
Hexasialo-Tf	2.615	2.69	2.881	2.86	3.009	4.13	3.206	2.73
CDT (asialo-Tf + disialo-Tf)	0.873	8.55	3.852	3.75	0.923	6.65	4.311	3.43

[a] Data of the first aliquot of the four samples analyzed each day during the 20-day evaluation period. ND refers to not detected.

study, reagents were changed on days 5, 11 and 17 and the data of all 20 days were found to be acceptable. The study, i.e. the stability of the system, was surveyed with control charts that included the mean value with two, three (warning limit) and four (out-of-control limit) SDs and every 5 days the mean value and the corresponding SD were recalculated for each chart from all acceptable data collected thus far. The study, however, could not be carried out without unexpected events. A total of three capillaries were used. Due to sticking of the cap of the waste vial to the interface block, the capillary broke on days 3 and 9 and had to be replaced. Mechanical problems with the sample garage led to the restart of sequences, namely on days 11 (three times), 12 (once), 13 (once), 17 (twice), 18 (once), 19 (once) and 20 (twice). The garage was replaced after completion of the study. There was a failure of current during the analysis of three samples, a current leakage during the analysis of one sample and higher detection times of the Tf isoforms for one sample. These five samples were reanalyzed at the end of the sequence, i.e. the same day. Furthermore, isoform values were somewhat lower than usual on day 16 (the values of the two controls were between −2 SD and −4 SD), but no intervention was necessary.

3.2. Recognition and identification of the transferrin pattern

Recognition and identification of the peaks in the electropherograms that originate from Tf isoforms can be accomplished via assessment of the relative migration times of the Tf isoforms (particularly in relation to tetrasialo-Tf), analysis of the sample prior and after spiking with a known mixture of Tf isoforms and/or prior and after immunosubtraction of Tf, and enzymatic sequential cleavage of sialic acid residues with analysis of the whole range of samples [9,14,16]. Detection times of Tf isoforms were found to vary within a very small range only

Table 4
Inter-day reproducibility data for the detection times of transferrin isoforms ($n=20$)[a]

Tf isoform(s)	Sample low		Sample high		Control low		Control high	
	Mean (min)	RSD (%)	Mean (min)	RSD (%)	Mean (min)	RSD (%)	Mean (min)	RSD (%)
Asialo-Tf	ND	ND	6.128	1.35	ND	ND	6.126	1.16
Disialo-Tf	6.365	1.42	6.379	1.45	6.410	1.47	6.377	1.25
Trisialo-Tf	6.434	1.41	6.452	1.48	6.483	1.48	6.450	1.27
Tetrasialo-Tf	6.576	1.45	6.596	1.52	6.626	1.53	6.593	1.32
Pentasialo-Tf	6.684	1.48	6.703	1.55	6.735	1.57	6.670	1.36
Hexasialo-Tf	6.794	1.50	6.813	1.60	6.848	1.62	6.808	1.38

[a] Data of the first aliquot of the four samples analyzed each day during the 20-day evaluation period. ND refers to not detected.

(Tables 2 and 4, [9,16]) and can thus typically be employed for peak assignment. Unambiguous recognition of Tf peaks was accomplished via immunosubtraction in free solution using an anti-human Tf antibody. For that purpose, the ratio of the volumes of serum and antibody solution and the incubation conditions had to be optimized. A relative amount of antibody solution that was 25% of the investigated serum was found to be insufficient for a complete removal of the Tf isoforms. Small peaks of tetrasialo- and pentasialo-Tf were detected (Fig. 2A, fourth electropherogram from top). The same was true employing equal amounts of antibody solution and serum (Fig. 2A, third electropherogram from top). Complete immunosubtraction of Tf isoforms could be obtained using 80 μl of serum and 160 μl of antibody solution (Fig. 2A, second graph from the top) whereas a further increase of the relative amount of antibody solution did not provide any advantage (Fig. 2A, top electropherogram). To the contrary, higher amounts of antibody solutions led to electropherograms that were overloaded with γ-globulins. The impact of the incubation time interval was studied with the optimized serum/antibody

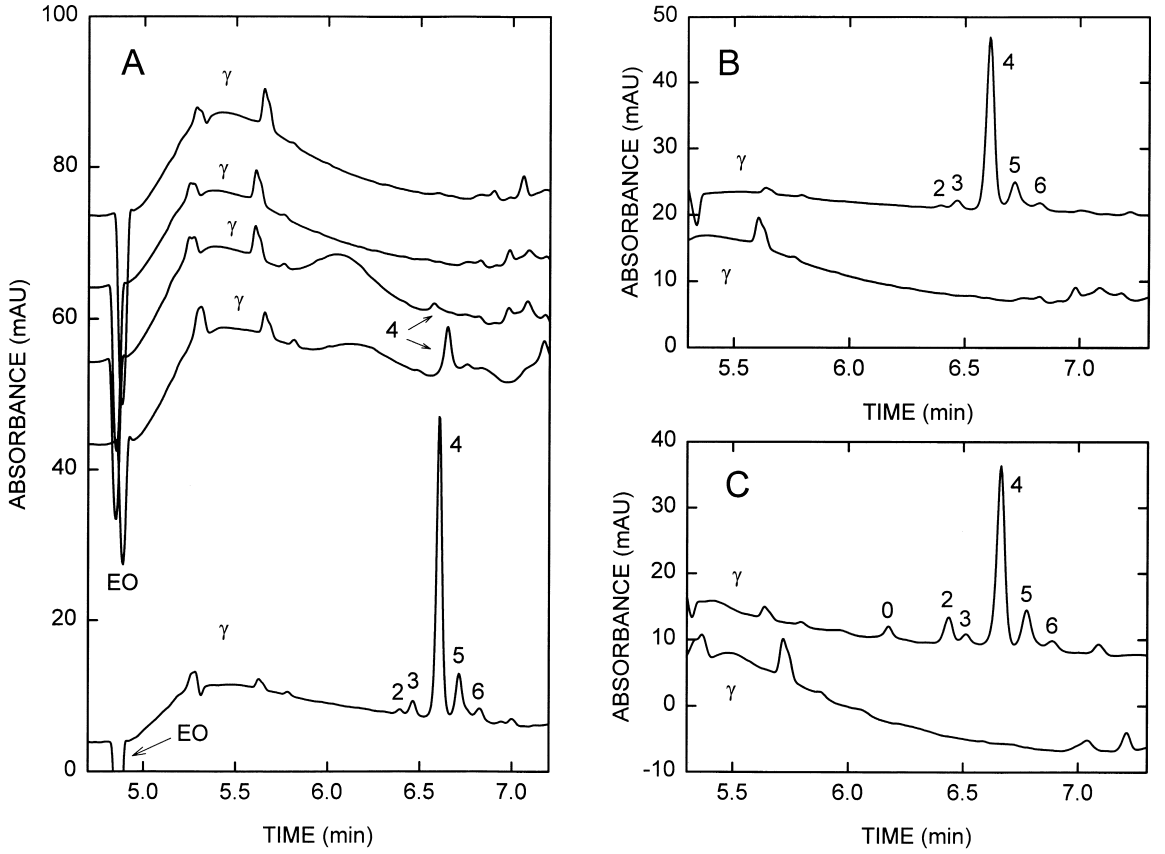

Fig. 2. Immunosubtraction of Tf. (A) Electropherogram obtained for a serum of a healthy individual without Tf antibody pretreatment (bottom graph) and after immunosubtraction using the serum/antibody solution proportions of 1:4, 1:2, 1:1 and 4:1 (v/v, from top to center, electropherograms plotted with different *y*-axis offsets). γ refers to the gamma region and EO marks the electroosmotic flow. (B) Electropherogram of a threefold diluted serum (top graph, presented with a *y*-axis shift of 7 mAU; serum dilution with two aliquots of ferric solution) of a healthy subject (CZE CDT value of 0.88%) compared to the data obtained after immunosubtraction of Tf with the reagent volume being twice that of the serum (bottom graph). (C) Electropherogram of an alcohol abuser (CZE CDT value of 13.3%, presented with a *y*-axis shift of 20 mAU) together with data obtained after immunosubtraction of Tf and the experimental conditions as for panel B. The numbers of all graphs refer to the detected Tf isoforms (for key see Fig. 1).

solution ratio. An incubation time of 1 h was found to be sufficient. No improvement was obtained with longer incubation times. However, an incubation of 15 min led to incomplete immunosubtraction (data not shown). Thus, Tf isoforms were immunosubtracted by having twice the volume of antibody solution compared to serum, an incubation at room temperature for 1 h and centrifugation for 20 min at 8000 *g* and 4 °C. Fig. 2B and C depict electropherograms obtained before and after immunosubtraction of Tf from the sera of a healthy subject and an alcohol abuser, respectively. The identification of the peak labeled as hexasialo-Tf was found to be somewhat difficult. In some sera the possibility of an interference in this part of the beta-region cannot be excluded (see Fig. 2B, bottom graph).

As has been discussed previously for the P/ACE 5500 [14,16] and also briefly for the P/ACE MDQ [9], neuramidase can be employed for the enzymatic cleavage of the terminal sialic acid residues of Tf. During hydrolysis, the higher sialylated Tf isoforms are gradually disappearing with time and new peaks are arising around and before the position of asialo-Tf as well as between asialo- and disialo-Tf (Fig. 3). The transient peak marked with ‘‘a’’ is presumably monosialo-Tf. It is shown to gradually increase within the first 20 h and 24 h for the cases of Fig. 3A and B, respectively. Then this peak is decreasing and

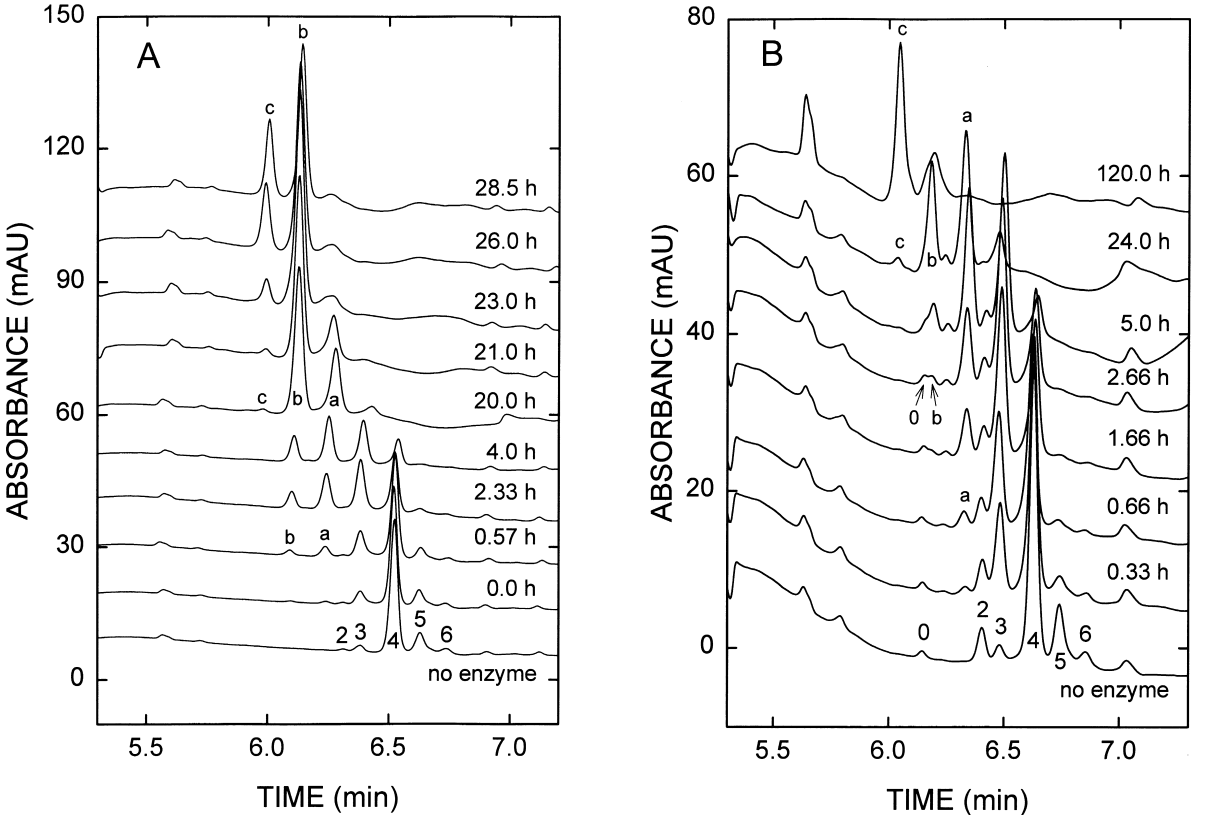

Fig. 3. Electropherograms obtained after enzymatic sequential cleavage of sialic acid residues from sera of (A) a healthy subject with a CZE CDT level of 0.76% and (B) an alcohol abuser with a CZE CDT value of 8.91% using (A) neuraminidase type X and (B) neuraminidase type III. The bottom graphs depict the patterns obtained without enzyme using the same amount of water instead of the enzyme solution. Consecutive electropherograms monitored at the indicated time intervals after commencement of the enzymatic incubation are presented with *y*-axis offsets of the *x*-axis adjusted for equal appearance of the corresponding peaks. The numbers of the bottom graphs refer to the detected Tf isoforms (for key see Fig. 1).

finally vanishing. Peaks ‘‘b’’ and ‘‘c’’ are assumed to be glycosylated and asialylated Tf isoforms. All these peaks can be immunosubtracted using an antibody against Tf and the patterns observed in our work are comparable to those reported by Legros et al. [16]. Asialo-Tf is not affected by the neuraminidase hydrolysis. This is nicely illustrated with the 0.33 h to 2.66 h time points of Fig. 3B. According to our data, asialo-Tf gradually becomes part of the growing peak ‘‘b’’. It is, however, not identical to the isoform of peak b. Using *N*-glycosidase F to cleave the glycans present in the isoforms of Tf, including those of peaks ‘‘b’’ and ‘‘c’’, and thereby producing asialo-Tf did not yet provide the anticipated data. Incubation of serum resulted in small amounts of asialo-Tf being formed only. Further efforts are required to investigate the processes leading to this final proof.

The screening of about 200 sera revealed five electropherograms with two major and a total of more than six Tf peaks (center graphs of Fig. 4), i.e. patterns that are clearly different compared to those normally observed with the sera of healthy subjects or alcohol abusers (Figs. 1, 2B and 2C). The area and heights of the two major peaks detected were found to be about half of the area and height of the tetrasialo-Tf peak of a normal serum pattern, thus supporting the hypothesis that the observed abnormal patterns belonged to genetic Tf variants. Human Tf is known to show genetic polymorphism caused by the substitution of one or more amino acids in the primary structure of the protein. Up to now, at least

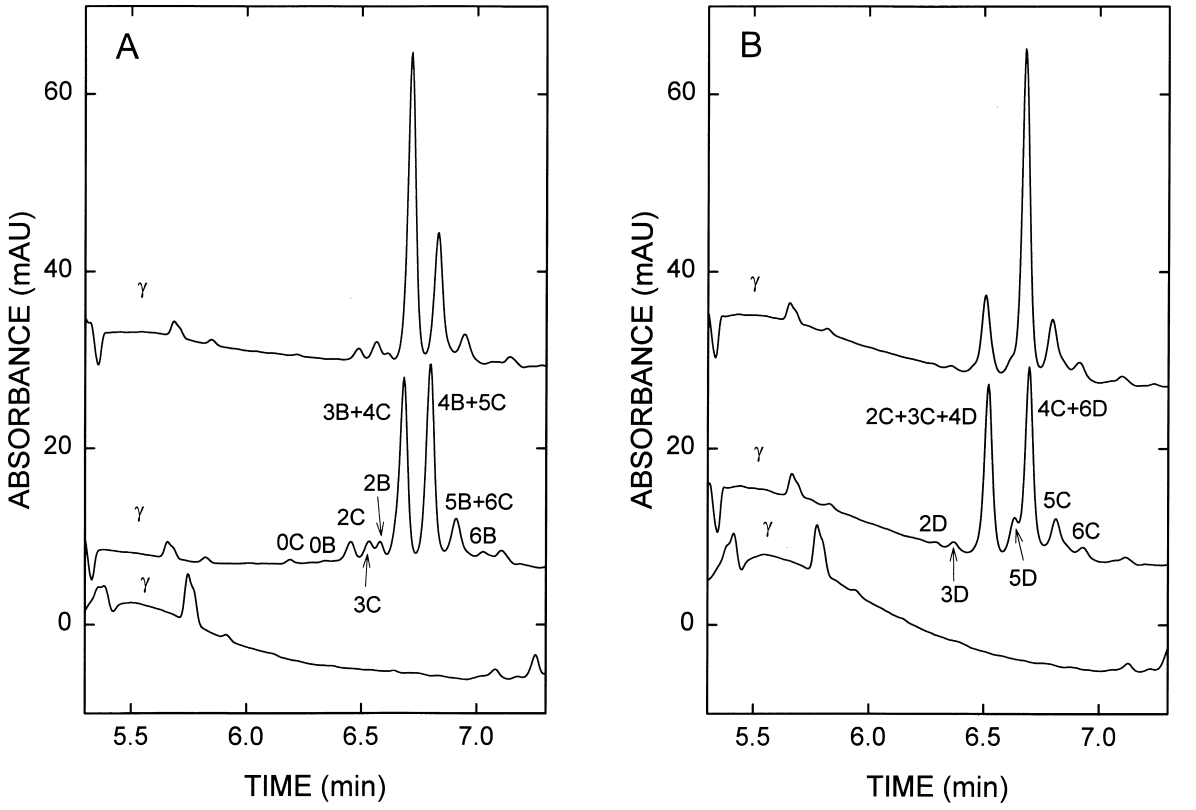

Fig. 4. Electropherograms obtained for (A) a heterozygous Tf-BC variant and (B) a heterozygous Tf-CD variant monitored without pretreatment (center graphs), after immunosubtraction of Tf (bottom graphs) and after spiking with the serum of a healthy individual (top graphs). Center and top graphs are presented with *y*-axis offsets of (A) 8 mAU and 27 mAU, respectively, and (B) 7 mAU and 27 mAU, respectively.

38 genetic Tf variants could be identified and only four of these occur with a prevalence of >1%. Tf variants can be divided into three groups. Tf-C, the most common phenotype in all populations, encompasses 16 distinct subtypes with slightly different isoelectric points, Tf-C1 being that with the highest prevalence (>95%) in Caucasians. Tf-B variants with lower p*I* values than Tf-C occur with lower frequencies, whereas genetic Tf-D variants having higher p*I* values are reported to be rare in Caucasians, but not in some other populations [6,25–27]. Closer inspection of the monitored electropherograms revealed the overall pattern as being a composite of two Tf patterns with an anodic time shift (shift to the right, center graph of Fig. 4A) or with a cathodic time shift (shift to the left, center graph of Fig. 4B). Immunosubtraction with the anti-human Tf antibody led to electropherograms with no peaks in the β-region (bottom graphs of Fig. 4) thereby proving the Tf origin of all the monitored peaks. Furthermore, in order to distinguish between the Tf isoforms normally observed and those shifted to the right or to the left, the sera were spiked with the control serum of a healthy individual and reanalyzed (top graphs of Fig. 4). B and D Tf variants could thereby be identified. In four of the five cases, the major peak of the atypical Tf isoforms, considered to be tetrasialo-Tf, eluted about 7 s after the corresponding peak of the common C-tetrasialo-Tf, thus indicating a shift to the anodic side due to an increase of the electrophoretic mobility (decrease of p*I*) of the unexpected isoforms (Fig. 4A, top graph) and the presence of genetic Tf-B variants in these cases originating from Tf-BC heterozygote individuals. All peaks present in the electropherograms could be unambiguously assigned either to a single isoform of one of the two genetic Tf alleles (B- or C-Tf variant) or to comigrating isoforms of the two Tf variants (Fig. 4A). In one case, a shift of the unusual Tf isoforms of about 10 s to the cathodic side was observed (top graph of Fig. 4B). Tf isoforms with shorter migration times compared to those of the common Tf-C can be attributed to the presence of a rare genetic Tf-D variant with isoforms having higher isoelectric points. All peaks could be allocated to single or unresolved isoforms of the Tf-C and Tf-D alleles (Fig. 4B). Although the number of selected individuals in our investigation is too small and heterogeneous to report the prevalence of Tf-BC (about 2%) and Tf-CD (about 0.5%) heterozygotes, it can be stated that the occurrence of Tf variants is not negligible. These findings are in agreement with the literature [6,26,27] and of importance for the determination of CDT for identification of alcohol abusers.

In Ref. [27] it was shown that BC and CD heterozygotes regularly led to low and high CDT results, respectively, when analyzed by any of the commercially available immunoassays, with the consecutive possibility of incorrect classification and misinterpretation of the test result. This is also true for the two samples depicted in Fig. 4A and B for which the %CDT TIA assay revealed CDT values of 4.6% and 2.9%, respectively. These values are higher than the reference value of 2.6%. Using CZE, the elevated CDT value of the serum of Fig. 4A could be confirmed. The CZE CDT level was estimated to be 8.28%, a value that was composed of the peaks of 0C and 0B and twice the peak 2C (0C+0B+2×2C). For the serum of Fig. 4B, however, the CZE CDT value of 0.84% (estimated to represent twice the peak value of 2D) is clearly comparable to those of individuals without elevated CDT levels (Figs. 1–3). In analogy to HPLC [27], CZE is shown to permit proper classification of the CDT patterns of BC and CD heterozygotes alike.

3.3. Recognition and identification of non-transferrin peaks

From the ~200 sera analyzed, selected electropherograms were found to comprise a peak of varying magnitude that was detected just prior to disialo-Tf (Fig. 5A). Immunosubtraction of Tf isoforms and reanalysis revealed that this peak did not belong to Tf (Fig. 5B, second graph from bottom). The manufacturer of the CEofix CDT-kit indicated that CRP could be responsible for that peak. Thus, immunosubtraction experiments with an anti-human CRP antibody were performed. In a first approach, 135 μl serum containing a large amount of CRP (120 mg/l, top graph of Fig. 5B) were incubated with 15 μl anti-CRP antibody solution for 30 min at room temperature. After centrifugation, 60 μl supernatant were saturated with 60 μl of ferric solution. Analysis of this sample revealed a decreased peak of interest

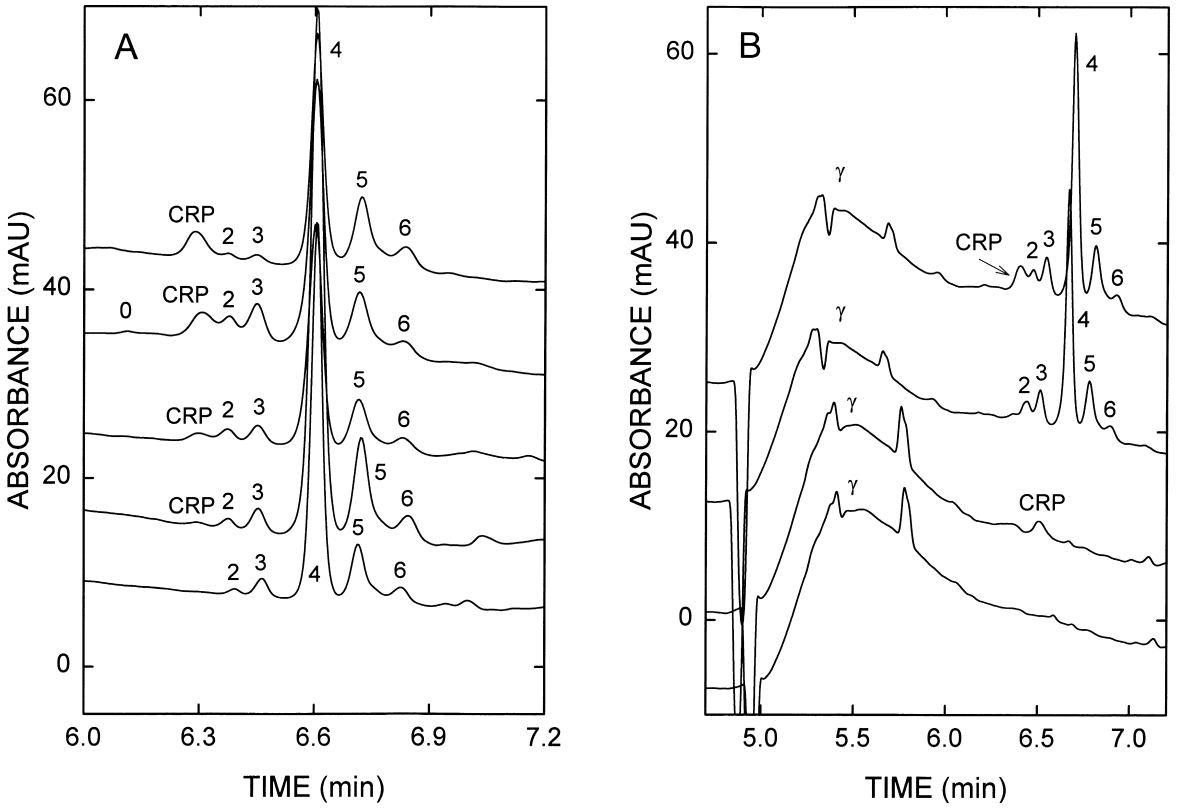

Fig. 5. Electropherograms before and after immunosubtraction of CRP. (A) Patterns of sera having undetectable CRP, 10 mg/l, 34 mg/l, 120 mg/l and 141 mg/l CRP (from bottom to top, respectively; data presented with *y*-axis offsets of *x*-axis adjusted for equal appearance of tetrasialo-Tf). (B) Electropherograms of a patient serum containing 120 mg/l CRP, of the same serum after immunosubtraction of CRP using 10% antibody solution, of the same serum after immunosubtraction of Tf, and of the same serum after immunosubtraction of CRP and Tf (from top to bottom, respectively; data presented with *y*-axis offsets). The CRP values are those determined with the immunoassay.

and an increase of the peak of disialo-Tf (Fig. 5B, second graph from top). The cause for the latter fact could be revealed as originating from a component in the anti-CRP antibody solution that was found to comigrate with disialo-Tf. The use of a higher amount of antibody solution led to a complete removal of the CRP peak together with an increased interference with disialo-Tf. For sera with smaller amounts of CRP, 5% (v/v) antibody solution was found to provide a complete removal, whereas with 2% (v/v) or less insufficient immunosubtraction of CRP was obtained. No improvement was noted with incubation times longer than 1 h and by incubating the serum with anti-CRP antibody in the presence of iron (data not shown). A procedure for the sequential removal of CRP and Tf isoforms in the same serum was developed. In the first step, CRP was removed employing 5 or 10% (v/v) of the anti-human CRP antibody solution, depending on the amount of CRP present in the serum. One part of the supernatant (50 μl) was saturated with an equal amount of ferric solution and analyzed (Fig. 5B, second graph from top). The other part of the supernatant (60 μl) was used for immunosubtraction of Tf (as described in Section 2.6). After sequential immunosubtraction, CRP and Tf were no longer detected (Fig. 5B, bottom graph).

The CRP peaks in the electropherograms amounted up to 5756 area units (top graph of Fig. 5A). The CRP level in the four patient sera of Fig. 5A and 14 other sera was determined using a commercial immunoassay with a detection limit of

3 mg/l (normal CRP range: <5 mg/l) and revealed CRP levels between 4 and 141 mg/l. Regression analysis of the peak areas in the electropherograms (between 433 and 5756 area units) with the immunoassay data resulted in a linear relationship ($y = 289.1 + 43.5x$ where x represents the immunoassay CRP data) with the correlation coefficient r being 0.962. Furthermore, for four sera whose CRP levels could not be determined with the immunoassay (CRP <3 mg/l), small peaks (peak areas: 176–831) were detected in the electropherograms. Thus, the CZE assay appears to be sensitive enough to determine elevated CRP levels in human serum. Based on our current experience, CRP levels up to about 70 mg/l were found not to interfere with CDT determination. With higher values, resolution between CRP and disialo-Tf was noted to become <1.24 and thus somewhat hampered the exact determination of disialo-Tf. Nevertheless, for the data presented in Fig. 5A, classification by CZE revealed data that were in agreement with those assessed by the immunoassays. Elevated CDT values were noted for the sera containing 34 and 120 mg/l CRP (CZE CDT values of 2.36% and 2.06%, respectively, and %CDT values with the older immunoassay of 9.1% and 8.0%, respectively) and normal CDT values were obtained for the sera with 10 and 141 mg/l CRP (CZE CDT values of 1.03% and 0.50%, respectively, and %CDT values with the newer immunoassay of 1.4% and 1.9%, respectively).

Four patient sera analyzed with the CZE assay revealed an abnormal pattern in the γ-region in that a large peak (15 to 35 mAU) was detected in the time window between about 5.2 and 6.0 min (Fig. 6). In one case, the unexpected peak was interfering with a possible asialo-Tf (Fig. 6B). In another case, a broad peak encompassing the whole region of γ-globulins from 4.9 to 5.6 min was noted (Fig. 6C). Serum protein electrophoresis (Fig. 7) and immunofixation (see insets in Fig. 6) was performed in all cases for further investigation of the nature and the origin of the observed peaks. In two cases, the abnormal serum pattern could be attributed to a monoclonal gammopathie of an immunoglobulin of the type IgG. Subtype specification revealed IgG lambda for both (Figs. 6A, B and 7). The broad double peak in the serum of Fig. 6C could be identified as a biclonal gammopathie of two distinct immunoglobulins of the type IgG lambda (see small arrows in the inset), whereas the serum whose electropherogram is depicted in Fig. 6D was found to represent a monoclonal gammopathie of an immunoglobulin of the type IgM kappa. For the four sera, CZE is predicting a normal CDT value (0.63%, 0.51%, 0.67% and 0.52% for the data of panels A to D, respectively). Furthermore, a fair number of sera stemming from patients treated at the departmental hepatology outpatient clinic were found to contain a broad peak comigrating with the Tf isoforms whose width was larger than the entire Tf pattern and whose height is between 5 and 15 mAU. The origin of this abnormality and its influence on the CDT determination by CZE is currently under investigation and will be the subject of a forthcoming communication.

4. Conclusions

CZE with the dynamic CEofix double coating performed in the P/ACE MDQ permits the simultaneous, individual, quantitative determination of Tf isoforms in human serum and thus CDT. The precision performance assessed over a 20-day period according to the internationally accepted NCCLS EP5-A guidelines revealed the assay as being highly reproducible with within-run and total precision being dependent on the Tf isoform level and RSD values ranging between 2.2 and 14.4% (within-run precision) and 3.6 and 17.6% (total precision). Inter-day RSD values for asialo-Tf were noted to be between 9.8 and 11.5% and for disialo-Tf between 3.8 and 8.6%, whereas those for CDT levels of 0.88 and 4.31% of total Tf were determined to be 8.6 and 3.4%, respectively. RSD values for the isoforms with higher abundance, trisialo-Tf, tetrasialo-Tf, pentasialo-Tf and hexasialo-Tf, were found to be between 0.4 and 4.1%. Tf patterns are recognized via detection times of Tf isoforms (RSD <1.7%) and isoform distribution, immunosubtraction and enzymatic sequential cleavage of sialic acid residues. Furthermore, heterozygous Tf-BC and Tf-CD variants are assigned via spiking with a known mixture of Tf isoforms (e.g. the serum of a healthy Tf-C homozygote). Among the non-Tf peaks monitored, the CRP peak detected shortly before disialo-Tf

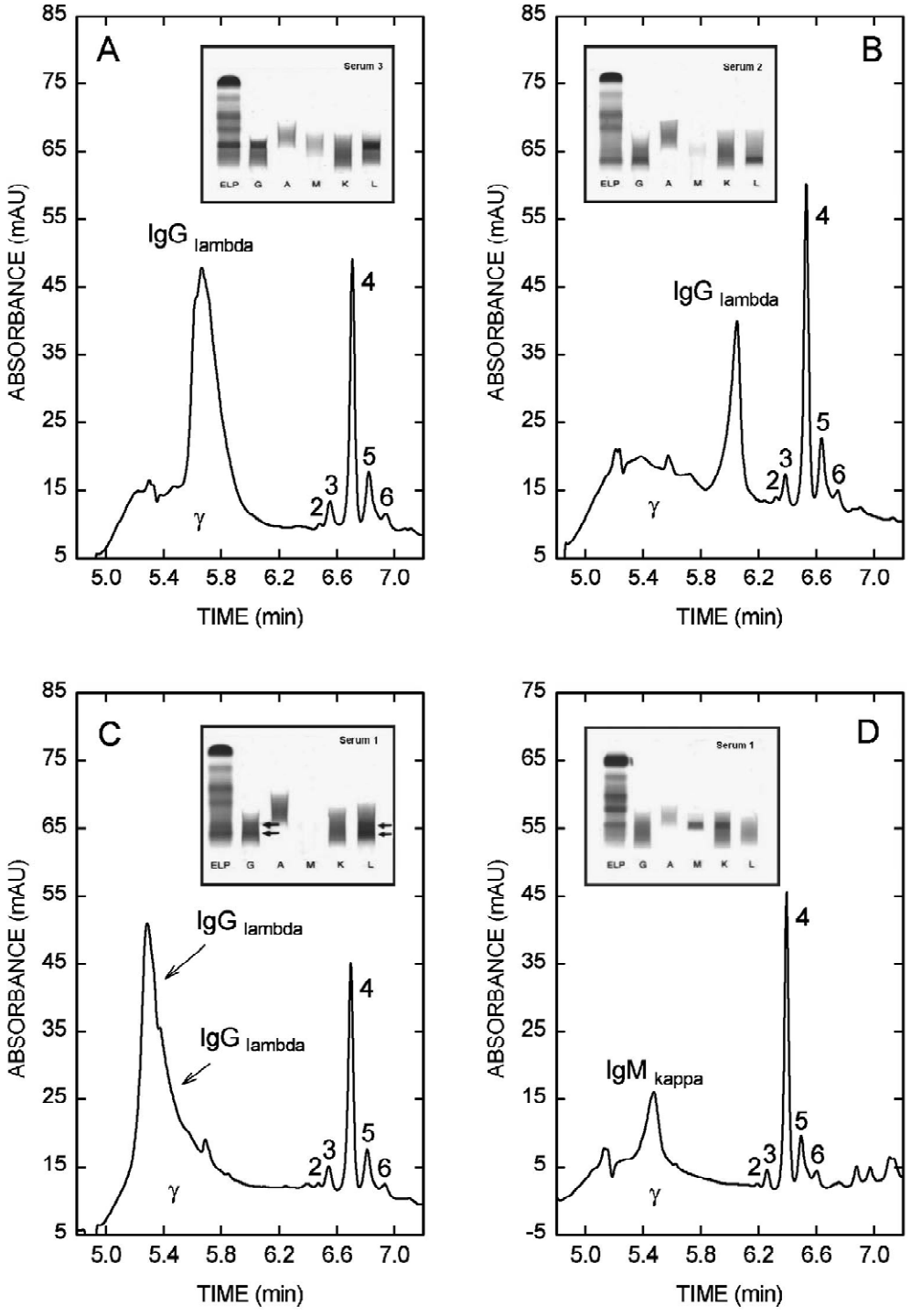

Fig. 6. Electropherograms with large, unusual peaks in the γ-region. The insets depict the immunofixation data.

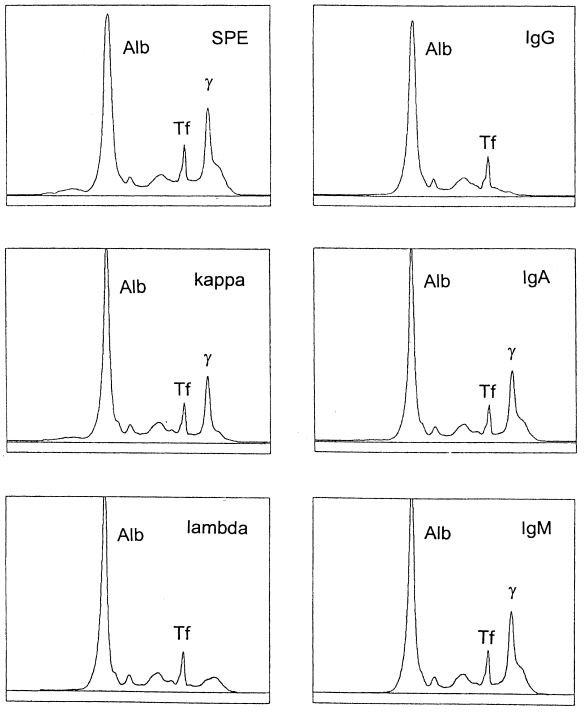

Fig. 7. CZE electropherograms for the serum of Fig. 6A without immunosubtraction (SPE trace, upper left panel) and with immunosubtraction prior to analysis (type of subtraction is mentioned in upper right corner of each panel). Alb, Tf and γ refer to albumin, transferrin and γ-region, respectively.

could be identified by immunosubtraction and CRP peak magnitudes were found to correlate well with immunochemically determined CRP serum levels. The CZE assay with dynamic double coating could thereby be shown to be sensitive enough to determine elevated CRP levels in human serum. Customary serum protein CZE, immunosubtraction CZE and immunofixation analyses can be employed for the identification of unusual peaks in the γ-region. Based on our work described in this paper and previously [9], as well as that of other laboratories [16], it is now clear that CZE with a dynamic double coating can reliably be employed for the determination of CDT in human serum. This simple, rapid and highly reproducible CZE assay is an attractive alternative to HPLC and can be employed for both screening and confirmation analysis. In analogy to HPLC, it has a high sensitivity (few false negatives) and a high specificity (few false positives) [16]. It is important to note that all data presented here were performed and evaluated according to the recommendations of the manufacturer of the CEofix CDT kit. Under these conditions, the separation of di- and trisialo-Tf is typically but not always >1.2 [9]. Thus, baseline resolution between these two peaks was not always obtained and baseline integration with mathematical deconvolution would possibly be a more appropriate data evaluation procedure. Alternatively, modifications of the electrophoretic configurations will have to be studied. Furthermore, before adoption to the routine arena, the reference limit or cut-off value for CDT will have to be established. Both of these aspects are currently being investigated in our laboratory.

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