ELSEVIER

Journal of Chromatography A, 979 (2002) 43–57

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Evaluation and optimization of capillary zone electrophoresis with different dynamic capillary coatings for the determination of carbohydrate-deficient transferrin in human serum

Christian Lanz[a], Martina Kuhn[a], Federica Bortolotti[b], Franco Tagliaro[b], Wolfgang Thormann[a,*]

[a]*University of Bern, Department of Clinical Pharmacology, Murtenstrasse 35, CH-3010 Bern, Switzerland*
[b]*University of Verona, Department of Public Medicine and Health - Unit of Forensic Medicine, Verona, Italy*

Abstract

Serum transferrin (Tf) comprises several isoforms with up to two complex oligosaccharide chains containing zero to eight sialic acid residues and neutral sugars. The major glycoform, known as tetrasialo-Tf, contains four sialic acid residues and accounts for about 80% of whole Tf in human serum. Carbohydrate-deficient transferrin (CDT) encompasses isoforms that are deficient in carbohydrate chains and consequently in sialic acid residues (including asialo-, monosialo- and disialo-Tf) and is a well known marker for chronic alcohol abuse. Recently capillary zone electrophoresis (CZE) has been reported as a tool extremely effective for the simultaneous, individual, quantitative determination of CDT isoforms. Three CZE methods that feature different dynamic capillary coatings were evaluated and optimized for CDT determination in human serum of alcohol abusers and control subjects. CZE separation was performed in alkaline borate buffers after serum sample saturation with iron, electropherograms were detected at 200 nm, data were evaluated as % area of disialo-Tf in relation to tetrasialo-Tf and peak identification was accomplished via relative migration times to tetrasialo-Tf, immunosubtraction and enzymatic sequential cleavage of sialic acid residues. Dynamic capillary coatings with diaminobutane, spermine and a double coating produced by commercially available proprietary agents were investigated and found to be suitable for determination of CDT in human serum. For all three approaches, best results were obtained in 50 μm I.D. fused-silica capillaries of 50 cm effective length and a capillary cartridge temperature of 20–25 °C. Using 3 m*M* 1,4-diaminobutane or 0.02 m*M* spermine in a borate-based running buffer of pH 8.3 provided data of remarkable similarity with resolution of di-, tri-, tetra- and pentasialo-Tf within 15–18 min. With the double coating, asialo-Tf and Tf isoforms with two to six sialic acid residues were baseline separated. Compared to the two amine-based procedures, the run times were found to be somewhat shorter, the detector signals higher, the applied power level significantly lower and the reproducibility better.

Keywords: Capillary electrophoresis; Coated capillaries; Transferrin; Protein

1. Introduction

Alcohol misuse and alcohol dependence are common in many different cultures and there are no

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

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01244-X

doubts that among the substances active on the central nervous system, ethanol has the heaviest medical, social and economic impact. Alcohol abuse can generally be divided in ''high risk drinking'' that means a regular daily intake of about 60 g or more of ethanol in males (about ≥40 g in females), and ''alcohol dependence'' which encompasses excessive drinking with consequential physical and psychological harm. Therefore, early diagnosis of alcohol abuse has become a very important topic not only in clinical medicine, but also in psychiatry, occupational, traffic and forensic medicine. In these fields, different questionnaires and biological markers like alanine aminotransferase (ALT), aspartate aminotransferases (AST), erythrocyte mean corpuscular volume (MCV), γ-glutamyltransferase (GGT), serum urate and triglycerides are currently used in assisting the effort of an objective and reliable diagnosis of alcoholism [1,2]. In the 1970s, Stibler et al. proposed carbohydrate-deficient transferrin (CDT) as a new biological marker for chronic or repeated excessive alcohol intake [3–5]. Transferrin (Tf), the most important iron transporting protein, is a glycoprotein with a molecular mass of about 79 570 that consists of a single peptide chain of 679 amino acids. The N-terminal (amino acids 1–336) and C-terminal (amino acids 337–679) domains are globular and encompass an iron binding site each. Tf has two N-linked carbohydrate chains at the positions 413 and 611 that are composed of a bi-tetraantennary carbohydrate chain containing *N*-acetylglucosamine, mannose, galactose and terminal sialic acid residues [6,7]. Based on a different content of end standing sialic acid residues (zero up to eight), human serum comprises different isoforms of Tf [1,7]. At alkaline pH, sialic acid residues are negatively charged and the Tf isoforms thus differ in their isoelectric points and their electrophoretic behavior. The major Tf isoform in humans consists of four sialic acid residues (tetrasialo-Tf) with an isoelectric point of 5.4 (after complete iron saturation). Following chronic alcohol ingestion of 50–80 g ethanol per day over a period of 1–2 weeks, the amounts of some less glycosilated forms with isoelectric points of 5.7 (disialo-Tf) and 5.9 (asialo-Tf) are reported to increase [1,2]. The majority of these isoforms were found to comprise one and no carbohydrate chain, respectively [8,9]. Isoforms with isoelectric points (p*I* values) ≥5.7, corresponding to molecules with two or less sialic acid residues, are collectively named CDT. CDT is considered to be the most specific marker available today for the detection of chronic, excessive alcohol intake [1].

CDT was first monitored by isoelectric focusing combined with immunofixation, zone immunoelectrophoresis or Western blotting. Later on, assays based upon ion-exchange and other forms of high-performance liquid chromatography were developed [1,2,10,11]. Sensitivity and specificity of these procedures were reported to be good. Productivity, however, is too low for routine use. Thus, various two-step assay kits based on the removal of non-CDT isoforms by ion-exchange chromatography on disposable microcolumns followed by immunochemical determination of the remaining Tf isoforms were developed. The commercially available kits differ mainly in the efficiency of the chromatographic separation step and in the immunochemical detection method (radioimmunoassay, enzyme immunoassay, immunoturbidimetric assay, etc), whereas the antisera used are in any case directed towards whole human Tf (no specificity for CDT). Because of insufficient resolution between isoforms with similar p*I*'s in the microcolumn chromatographic step, the composition of the collected CDT fraction not only encompasses CDT according to the above stated definition, but may also contain an unspecified amount of trisialo Tf. Another problem is the well-known susceptibility of specific (abnormal genetic Tf variants) and nonspecific interferences of the immunometric determination. CDT immunoassays are therefore prone to analytical inaccuracy [1,2,12–14].

During the last decade, a great deal of interest was focused on the determination of Tf isoforms and particularly CDT by capillary electrophoresis (CE) [15–29]. Capillary isoelectric focusing (cIEF) was found to be capable of resolving the major forms of human [15,16] and bovine [19] Tf. Due to the complexity and insufficient reproducibility of these approaches, however, cIEF could not be adopted for the determination of CDT in human serum. The use of free solution capillary zone electrophoresis (CZE) was more successful. Kilàr and Hjertén reported the resolution of the Tf isoforms with zero and up to six sialic acid residues in polyacrylamide-coated capillaries and with a borate/Tris buffer of pH 8.4

containing 0.3 m*M* EDTA [17]. Oda and Landers published a detailed study on the CZE separation of Tf isoforms from purified Tf of different species, including humans. Resolution of the major Tf isoforms was studied in bare fused-silica capillaries with borate and phosphate buffers containing different cationic buffer additives for column conditioning, including 1,4-diaminobutane (DAB) also referred to as putrescine or tetramethylenediamine, decamethonium and hexamethonium [18]. Employing bare fused-silica capillaries and a Tris/glutamic acid buffer at pH around 8.6, a CZE study of abnormal glycoforms of serum Tf in carbohydrate-deficient glycoprotein syndrome type I was published by Iourin et al. [20]. Prasad et al. [21] and Oda et al. [22] studied the CZE determination of CDT in DB-17 coated capillaries using borate buffers with different alkylated celluloses as additives. Significant improvement was achieved with respect to analysis time, but the procedure required immunopurification of serum before CZE. Furthermore, Trout et al. [27] and Beisler et al. [28] used fluorocarbon-coated capillaries achieving good resolution of the Tf isoforms. However, longevity of the covalently-coated capillary was too short and the separation performance was affected by an aging process of the capillary. Independently, Tagliaro et al. published a detailed investigation of the CZE separation of human Tf isoforms in bare fused-silica capillaries with I.D.'s of 20–100 μm combined with different buffers. Good separation within reasonable time and without complicated sample preparation was obtained with a short capillary (30 cm to the detector) of 20 μm I.D. [23]. Elongation of the capillary length and addition of 1.5 m*M* or 3 m*M* of DAB to a 100-m*M* borate buffer lead to further improvements [24,25] such that the assay could be introduced in Verona (Italy) for forensic confirmation of sera that were found to be positive using CDT immunoassays. The efficacy of DAB in preventing proteins to adhere to the capillary wall was also demonstrated by Giordano et al. who investigated the effect of increasing DAB concentrations (0–4 m*M*) added to a borate buffer in a 50-μm I.D. bare fused-silica capillary [26]. In agreement with the work from the laboratory in Verona, good separation of the Tf isoforms was achieved in presence of 3 m*M* DAB. Finally, Wuyts et al. [29] investigated the analysis of CDT with proprietary reagents that provide a dynamic double coating of the capillary produced by adsorption of a polycation prior to application of a second layer composed of a polyanion. This coating procedure is comparable to that described by Katayama et al. [30]. Unfortunately, as disialo- and trisialo Tf were not separated with the buffer used, this assay cannot be employed for the determination of CDT [31].

The main problem in CZE of proteins is the interaction of the highly charged proteins with the silanol groups of the capillary wall, resulting in a strong impairment of separation performance. Different approaches to overcome protein adsorption have been proposed, including the use of electrolyte solutions at extreme pH values, buffers of high ionic strength, buffer additives that dynamically coat the capillary wall and covalently coated capillaries [32,33]. Based upon the recent literature [15–29], the use of buffer additives that provide dynamic capillary coatings appears to be the most promising approach for analysis of CDT in human serum. The aim of this work was: (i) to revisit and thereby optimize the use of DAB [24–26] as capillary conditioner; (ii) to test the employment of spermine [*N*,*N*′-bis(3-aminopropyl)1,4-butanediamine] as alternative buffer additive, an oligoamine that is known to be an effective quencher of protein adsorption [32,34] and that has been successfully employed for CZE analysis of ovalbumin glycoforms [35]; and (iii) to evaluate a newly developed commercial kit that provides a dynamic double coating (CEofix CDT-kit of Analis, Namur, Belgium). The performance of the three dynamic coatings were studied in terms of resolution of Tf isoforms, run times, signal magnitudes and reproducibility. For optimization, capillary dimensions, buffer composition, amount of sample injected, applied power and capillary cartridge temperature were varied. The ultimate goal of this study was to find the most suitable approach for the routine arena.

2. Experimental

2.1. Chemicals

If not stated otherwise, chemicals were of ana-

lytical grade. Disodium tetraborate decahydrate and sodium hydrogen carbonate were from Merck (Darmstadt, Germany). Caffeine was obtained from Inselspital-Apotheke (Bern, Switzerland). Ferric(III) chloride hexahydrate and spermine tetrahydrochloride (≥99.5%) were purchased from Fluka (Buchs, Switzerland). DAB (approximately 98%), neuraminidase type X from clostridium perfringens (210 units/mg solid, 240 units/mg protein) and iron saturated holo-Tf (minimum 98%) were from Sigma (St. Louis, MO, USA). Rabbit anti-human Tf antibody (titer: 2800 mg/l) was obtained from Dako (Glostrup, Denmark). Buffers and reagents of the Analis CEofix-CDT-kit for quantitation of CDT with the Beckman Coulter P/ACE MDQ (kit No. 10-004740) were kindly provided by Analis. It is important to note that this kit comprises a similar but not identical buffer than that used by Wuyts et al. [29].

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 were drawn by venipuncture and collected in native plastic tubes without additives (Monovette, white cap, Sarstedt, Sevelen, Switzerland). After clotting the blood samples were centrifuged for 10 min at 2000 g at room temperature. 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. For the experiments with DAB and spermine, 200 μl of serum was combined with 5 μl of an aqueous 10 m*M* ferric(III) chloride solution and 5 μl of a 500 m*M* solution of sodium hydrogen carbonate in a plastic vial. After vortex mixing for 30 s the sample was incubated at room temperature for 30 min. Then, 5 or 10 μl of the sample was transferred into a sample vial and, if not stated otherwise, diluted 10-fold with water. The solution was gently mixed by aspiration and release of part of the fluid with a plastic pipette. For the determination of the electroosmotic flow (EOF), an aqueous caffeine (100 μg/ml) solution was used to dilute the iron saturated serum. For the experiments with the Analis CDT-kit, 60 μl of serum and 60 μl of the ferric solution of the kit were mixed directly in the microtiter plate (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions. The sample was mixed gently by aspiration and release of part of the fluid with a pipette. No incubation time was required to saturate the serum with iron.

2.4. Buffer preparation

For the experiments with DAB, borate stock solutions with concentrations of 12.5–200 m*M* tetraborate and a pH of 8.3 (adjusted via addition of 6 *M* HCl) and a 90 m*M* stock solution of DAB containing 0.05 m*M* HCl were prepared and kept at 4 °C. The running buffers with the desired concentrations of tetraborate and DAB were prepared daily. Similarly, for the experiments with spermine, pH 8.3 borate stock solutions (25, 50 and 100 m*M* tetraborate) and two stock solutions comprising 50 and 1 m*M* spermine, respectively, were prepared and stored at 4 °C. The running buffers were prepared daily. The higher concentrated stock solution of spermine was used for preparing buffers with spermine concentrations from 3.0 to 0.1 m*M*, whereas the 1 m*M* stock solution was employed to obtain spermine concentrations between 0.05 and 0.01 m*M*. For the experiments with the double coating, the buffers were employed as supplied in the kit.

2.5. Instrumentation and running conditions for the experiments with DAB and spermine

CZE measurements were performed on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter) which was equipped with fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 15, 25, 50 and 75 μm I.D. and 60.2, 50.2 and 40.2 cm (50.0, 40.0 and 30.0 cm to the detector) total length. All capillaries had an O.D. of about 375 μm. If not stated otherwise, the temperature controls of the capillary cartridge and the sample tray were set to 25 °C and 20 °C, respectively, samples were introduced by positive pressure and the applied

voltage was 20 kV. Reversed polarity (cathode at the injection end) was used for the experiments with spermine concentrations ≥0.5 m*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).

New capillaries were sequentially rinsed with 1 *M* NaOH, 0.2 *M* NaOH, water and running buffer (90 min each for capillaries of 15 and 25 μm I.D., 20–30 min each for capillaries 50 and 75 μm I.D.) by applying a pressure of 60 p.s.i. (1 p.s.i. ≡6894.76 Pa) at the inlet end. Then the capillaries were filled with running buffer and kept at room temperature for 60 min followed by application of 20 kV for 30 min. At the beginning of a day, capillaries were typically rinsed for 5–6 min with 1 *M* NaOH with a pressure of 60 p.s.i. from the inlet side, followed by rinsing for 10 min with 0.2 *M* NaOH and for 12–15 min with running buffer with a pressure of 60 p.s.i. from the outlet side. The conditioning step at the beginning of the day was completed by application of 20 kV for 10–15 min. Between runs, capillaries were rinsed from the outlet side with 0.2 *M* NaOH at 60 p.s.i. for 4 min (15 and 25 μm I.D.) or 2 min (50 and 75 μm I.D.) and with running buffer at 30–40 p.s.i. for 6 min (15 and 25 μm I.D.) or 3 min (50 and 75 μm I.D.). At the end of the day, the capillaries were first washed with 1 *M* NaOH from the outlet side followed by rinses from the inlet side with 1 *M* NaOH, 0.2 *M* NaOH and water (60 p.s.i., 10 min each with 15 and 25 μm I.D., 5 min each with 50 and 75 μm I.D.). Capillaries and their ends were kept in water during storage.

2.6. Instrumentation and running conditions for the experiments with the double coating

If not stated otherwise, CZE measurements were performed according to the instructions of the manufacturer of the CEofix CDT-kit (Analis) on the P/ACE MDQ instrument (Beckman Coulter). The system was equipped with a fused-silica capillary (Analis) of 50 μm I.D. and 60.2 cm (50.0 cm length to the detector) total length. The O.D. was 360 μm. The temperature controls of the cartridge and sample tray were set to 40 °C and 15 °C, respectively. New capillaries were rinsed for 10 min with 0.2 *M* NaOH by applying a pressure of 40 p.s.i. at the inlet end. The same washing step was employed at the beginning of each day. At the end of a day the capillary was rinsed for 5 min with 0.2 *M* NaOH and for 5 min with water by applying a pressure of 60 p.s.i. at the inlet end. Capillaries were stored wet with the ends kept in water. Between runs capillaries were rinsed with 0.2 *M* NaOH for 1 min with a pressure of 60 p.s.i. from the inlet end and then for 1.5 min with 0.2 *M* NaOH by applying simultaneously a constant current of 80 μA and a pressure of 20 p.s.i. at the inlet end. At the beginning of a new run, the capillary was rinsed from the inlet to the outlet side for 1.5 min with the initiator buffer and for 2 min with the run buffer of the CDT kit by applying a pressure of 15 p.s.i. Sample was introduced by applying a vacuum of 0.3 p.s.i. for 10 s at the outlet side. A voltage of 28 kV was applied (anode at the injection end) and the current was about 26 μA. Detection and data processing was performed as in Section 2.5. Selected experiments were performed with lower cartridge temperature and/or increased capillary length.

2.7. Evaluation of electropherograms

Data evaluation was based upon peak area measurements. For comparison of all approaches described in this manuscript, the amount of CDT was evaluated as % area of disialo-Tf in relation to tetrasialo-Tf and referred to as Tf-index [25]. Resolution (R_s) was calculated with the 32 Karat Software (Beckman Coulter) using $R_s = 1.18(t_2 - t_1)/[(W_{h/2})_1 + (W_{h/2})_2]$ where t_i and $(W_{h/2})_i$ represent the detection time and peak width at half height of firstly ($i=1$) and secondly ($i=2$) detected peak, respectively. An R_s value of ≥1.4 represents baseline resolution. Peak asymmetry (tailing factor, defined as half of the peak width at 5% of peak height divided by the distance from the peak maximum to the leading edge of the peak at the position of 5% peak height) and efficiency expressed in plates/m were also determined with this software.

2.8. Immunosubtraction and enzymatic digestion

For immunosubtraction of Tf, 80 μl of serum was vortex mixed in a plastic vial with 160 μl of anti-

human Tf immunoglobulins and incubated at room temperature for 1.5 h. After centrifugation at 10 000 *g* for 25 min at 4 °C, 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 digestion with neuraminidase, the enzyme was reconstituted to 1 U/ml with 50 m*M* sodium acetate (pH 5.0, adjusted with acetic acid). Serum (160 μl) was saturated in a plastic vial with 160 μl of the ferric solution of the CEofix CDT-kit and combined with 160 μl of the enzyme solution. After vortex mixing, the sample was first incubated at room temperature for 5 h and then at 37 °C until about 72 h. Aliquots were analyzed with the double coating method.

2.9. CDT determination with the %CDT TIA test

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.

3. Results and discussion

3.1. Experiments with DAB

The basis for the experiments with DAB was the work of Tagliaro et al. [24,25] in which CDT was analyzed in 20 μm I.D. capillaries employing a borate buffer of pH 8.3 containing 1.5–3 m*M* DAB. The performance of DAB was further investigated in capillaries of different inner diameters (15, 25, 50 and 75 μm, Fig. 1) and by varying borate concentration, applied voltage, operational temperature and amount of sample injected. Peak identification of the minor isoforms was accomplished by calculating the relative migration times to tetrasialo-Tf. Iron saturated sera were diluted 10-fold with water or an aqueous caffeine solution (100 μg/ml). Selected electropherograms obtained for the serum of a healthy subject and using capillaries of 50 cm effective length are presented in Fig. 1.

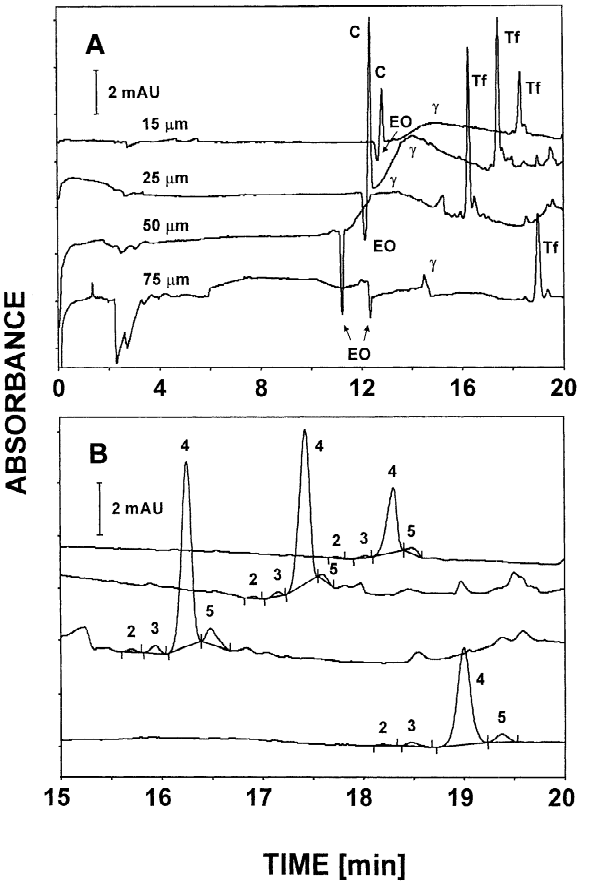

Fig. 1. Optimized CZE electropherograms of a serum from a healthy individual using borate buffers of pH 8.3 with DAB in capillaries of 15, 25, 50 and 75 μm I.D. (graphs from top to bottom) and 50 cm effective length. The entire electropherograms and the sections with the Tf pattern on an elongated *x*-axis scale are presented in panels A and B, respectively. The DAB concentrations were 2.25, 2.25, 3.0 and 3.0 m*M*, respectively, and the borate concentrations were 100, 100, 50 and 25 m*M*, respectively. The injection times (applied pressure) were 70 s (0.5 p.s.i.), 19 s (0.5 p.s.i.), 4 s (0.5 p.s.i.) and 5 s (0.2 p.s.i.), respectively. The applied voltages were 20 kV (I.D. <75 μm) and 18 kV (I.D. $=75$ μm) and the currents were 10.0, 30.6, 57.4 and 78.0 μA, respectively. The temperature of the cartridge was 25 °C in all cases. Other conditions are listed in Table 1. For the sake of clarity, electropherograms are presented with *y*-scale offsets. Key: 2, disialo-Tf; 3, trisialo-Tf; 4, tetrasialo-Tf; 5, pentasialo-Tf; C, caffeine; γ, gamma region; EO, electroosmosis.

Using a 25-μm I.D. capillary, 2.25 m*M* DAB in a 100 m*M* borate buffer, a voltage of 20 kV (current

about 30 μA) and a cartridge temperature of 25 °C, nice resolution of the major Tf isoforms, including di- and trisialo-Tf, was obtained within 18 min, whereas pentasialo-Tf was only partially separated from tetrasialo-Tf (second graph from top in Fig. 1A and B, Table 1). Asialo-Tf, present in sera from alcohol abusers, was not detected (data not shown), and hexasialo-Tf could not be identified. Depending on the injection time which was varied between 13.5 and 30 s (0.5 p.s.i. pressure, plug ≤0.76% of capillary volume), the height of the tetrasialo-Tf peak was 2.5–7 mAU and the signal-to-noise ratio for disialo-Tf (smallest peak) was ≤6. The effect of cartridge temperature on the separation of Tf isoforms was investigated between 20 and 45 °C (in steps of 5 °C). The temperature has an influence on the viscosity and conductivity of the buffer system and thus the mobilities of the analytes and electroosmosis. With increased temperature, the power dissipation in the capillary (expressed in W/m, for selected values refer to Table 1) and the intracapillary temperature become higher and therefore may require lowering the voltage or decreasing the electrolyte concentration of the buffer. Detection time could be reduced from about 21 min at 20 °C to about 11 min at 45 °C (electropherograms not shown). For the entire temperature interval, the applied power and current increased from 0.93 to 1.48 W/m and from 28 to 44 μA, respectively. The separation characteristics did not alter much except that the resolution between di- and trisialo-Tf was found to deteriorate as function of temperature. A similar effect on the migration times was observed by increasing the voltage from 20 to 30 kV (in steps of 5 kV, temperature of 25 °C). The detection time could thereby be reduced from about 18 min to about 11.5 min. The current increased from 30 to 48 μA and the power applied changed from 1.02 to 2.38 W/m, indicating that the intracapillary temperature is increasing as well [36]. Despite of the relatively high power level associated with the application of 30 kV, the separation was found to be almost as good as that obtained with 20 kV and 25 °C and was clearly better than with 20 kV and 45 °C (data not shown). No further improvement of peak resolution was obtained by varying the borate concentration up to 200 m*M* and by dilution of the iron saturated sera 5-fold or 20-fold. Increased peak resolution between disialo- and trisialo-Tf was noted after reducing the I.D. of the capillary to 15 μm (top graphs in panels A and B of Fig. 1, Table 1). However, much smaller signals were observed and the signal-to-noise ratio for disialo-Tf became very low. Thus, no further work with 15 μm I.D. capillaries was undertaken.

Enlargement of the capillary I.D. lead to electropherograms with increased signal magnitudes

Table 1
Experimental conditions and resolution data of selected electropherograms

Fig.	Experimental conditions							Resolution		
	Coating agent	Agent concentration (m*M*)	Capillary I.D.[a] (μm)	Sample plug[b] (%)	Cartridge temperature[c] (°C)	Power level (W/m)	Run temperature[d] (°C)	Di-/tri-sialo-Tf	Tri-/tetra-sialo-Tf	Tetra-/pentasialo-Tf
1	DAB	2.25	15	0.63	25	0.34	27.2	2.472	1.870	0.977
1	DAB	2.25	25	0.48	25	1.02	31.5	1.755	1.583	0.958
1,2A	DAB	3.00	50	0.40	25	1.91	37.2	1.493	1.847	1.207
1	DAB	3.00	75	0.45	25	2.35	40.0	1.585	2.380	1.571
4	spermine	0.02	25	0.68	25	1.00	31.4	1.820	1.777	1.079
5A	spermine	0.02	50	0.40	25	1.89	37.1	1.334	1.898	1.335
6A	double coating	–	50	0.82	40	1.21	47.7	1.368	2.715	1.935
7A	double coating	–	50	0.82	40	1.21	47.7	0.781	2.421	1.640
7B	double coating	–	50	0.54	20	0.73	24.7	1.421	2.706	1.734
8	double coating	–	50	0.58	20	0.42	22.7	1.332	2.715	1.736

[a] Effective capillary lengths were 50 cm (Figs. 1, 2 and 4–7) and 70 cm (Fig. 8).
[b] Theoretical sample plug length (expressed in % of capillary length) calculated with the CE Expert, version 1, software (Beckman).
[c] Temperature of the circulating cooling fluid.
[d] Intracapillary temperature estimated based upon a temperature increase of 6.4 °C per applied 1 W/m [36].

(Fig. 1) and increased operational temperatures (Table 1). Best results were obtained with a 50 μm I.D capillary, an effective capillary length of 50 cm, a 50 m*M*, borate buffer of pH 8.3 containing 3.0 m*M* DAB, an applied voltage of 20 kV and an operational temperature of 25 °C (second graph from bottom in Figs. 1 and 2). For a pressure of 0.5 p.s.i., the injection time was optimized to 4 s, corresponding to a theoretical plug length of 0.40% of the capillary length. The electroosmotic mobility (m_{eo}) and applied power levels were calculated to be $2.24 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$ and 1.91 W/m, respectively, and the estimated intracapillary temperature was estimated to be 37.2 °C. Under these conditions, the Tf isoforms

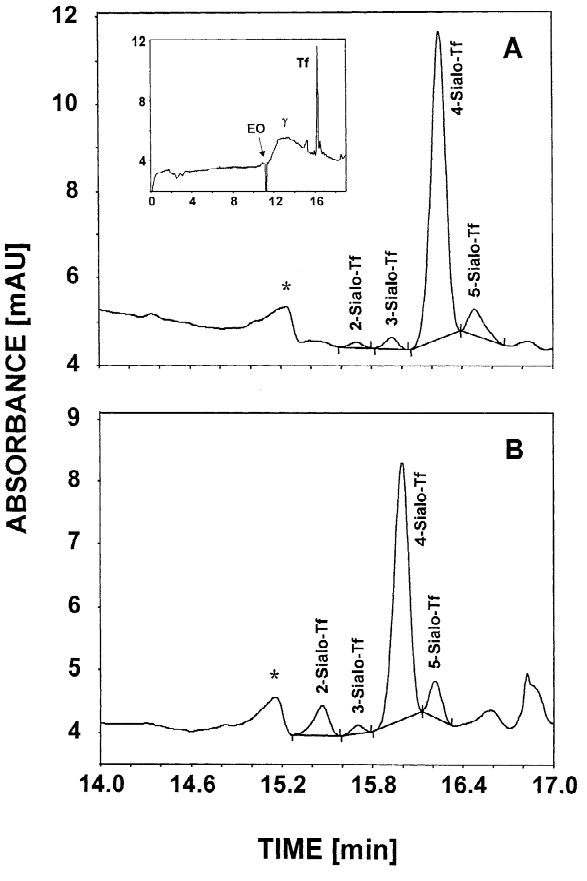

Fig. 2. Electropherograms in presence of 3.0 m*M* DAB obtained with (A) a serum of a healthy individual and (B) a serum of an alcohol abuser in a 50 μm I.D. capillary of 50 cm effective length. Other experimental conditions as for the 50 μm I.D. electropherogram of Fig. 1. The insert in panel A depicts the entire electropherogram. Key as for Fig. 1.

were detected within 17 min. Baseline separation of di- and trisialo-Tf was achieved and the resolution between tetrasialo-Tf and pentasialo-Tf was good (Table 1). The height of the tetrasialo-Tf peak was about 7 mAU and the signal-to-noise ratio for disialo-Tf in the pattern of healthy individuals (Figs. 1 and 2A) was between 10 and 15. Except for peak resolution, no further improvement was obtained by enlarging the I.D. of the capillary to 75 μm (bottom graphs in Fig. 1, Table 1).

The data presented in Fig. 2 represent typical serum Tf patterns of a healthy individual (Fig. 2A) and an alcohol abuser (Fig. 2B). Data were evaluated as % area of disialo-Tf in relation to tetrasialo-Tf and referred to as Tf-index, the normal value assessed in 20 μm I.D. capillaries being ≤2.27% [25]. The two Tf-indices were thereby calculated to be 1.35 and 12.06%, respectively. Thus, CZE with DAB as capillary conditioner was found to correctly classify the two sera. The same was found to be true for the %CDT TIA test that revealed values of 2.0 and 6.3%, respectively. It is important to note, that asialo-Tf could not be detected with the CZE assay (Fig. 2B). Furthermore, hexasialo-Tf could not be identified. The peaks marked with asterisks are of unknown origin and were present in most electropherograms (for exceptions refer to Fig. 1). Typical reproducibility data for peak efficiency, peak shape and resolution obtained via analysis of a serum of a healthy individual are summarized in Table 2, whereas corresponding RSD values for detection time, peak area and Tf-index are presented in Table 3.

3.2. Experiments with spermine

The effect of varying spermine concentrations on electroosmosis and the separation of Tf isoforms was investigated in capillaries of 25 μm I.D., 50 cm effective length and a cartridge temperature of 25 °C. First, a solution of purified human holo-Tf (0.5 mg/ml in water) and iron saturated serum of a healthy person that was diluted with an aqueous solution of caffeine (100 μg/ml) were analyzed employing 100 m*M* borate buffers of pH 8.3 comprising spermine concentrations between 3.0 to 0.02 m*M*. Selected electropherograms are presented in Figs. 3 and 4. For monitoring of electroosmosis in

Table 2
Typical peak efficiency, peak shape and resolution data obtained for disialo-Tf of a serum of a healthy individual[a]

Dynamic coating	Cartridge temperature (°C)	Efficiency		Asymmetry		Resolution di-/trisialo-Tf	
		Mean (plates/m)	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
Intra-day ($n=3$)							
DAB	25	199 286	32.78	1.15	28.96	1.36	6.74
Spermine	25	163 036	33.43	0.95	0.95	1.37	13.66
Double coating	40	369 245	4.01	0.92	3.06	1.24	2.75
Double coating	20	316 838	3.71	0.97	7.15	1.35	3.53
Inter-day ($n=5$)							
DAB	25	261 385	28.17	1.12	34.42	1.45	10.62
Spermine	25	242 806	29.15	1.11	21.25	1.50	15.68
Double coating	40	377 274	4.84	0.90	1.39	1.26	2.23
Double coating	20	338 277	8.59	0.92	4.13	1.35	3.03

[a] For examples of electropherograms and conditions refer to panels A of Figs. 2, 5, 6 and 7.

absence of proteins, caffeine (1 mg/ml in water) was injected. With spermine concentrations between 3.0 and 0.5 m*M* and application of 20 kV, caffeine could not be detected within 90 min of current flow. This means that under these conditions the EOF was markedly reduced, abolished or even reversed. The data suggested that $m_{eo} < 0.28 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. Similarly, from experiments with reversed polarity, it could be concluded that $m_{eo} > -0.40 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. For detection of Tf the polarity had to be reversed (−20 kV, first and second graphs from top of Fig. 3, top graph of Fig. 4). Under these conditions highly charged anions are reaching the detector first. For analysis of serum in presence of 3.0 m*M* spermine, a large splitted peak representing albumin was detected at 18 min, followed by several small peaks probably encompassing α1- and α2 globulins and finally Tf at about 27 min (top graph of Fig. 4). No separation between disialo- and trisialo-Tf was obtained. Shortening of the capillary or application of a higher voltage did not lead to improved Tf isoform separation (data not shown). The same was found to be true with a 0.5 m*M* spermine concentration (second graph from top in Fig. 3).

Table 3
Typical reproducibility data obtained for disialo-Tf and tetrasialo-Tf of a serum of a healthy individual[a] (RSD values in %)

Dynamic coating	Cartridge temperature (°C)	Detection time		Relative detection time Di-/tetrasialo-Tf	Relative peak area[c]		Tf-index[b]
		Disialo-Tf	Tetrasialo-Tf		Disialo-Tf	Tetrasialo-Tf	
Intra-day ($n=3$)							
DAB	25	1.11	1.13	0.11	14.65	4.50	14.91
Spermine	25	8.08	8.50	0.41	43.60	43.81	9.93
Double coating	40	0.04	0.06	0.04	3.73	6.57	3.37
Double coating	20	0.02	0.02	0.02	3.68	2.63	2.53
Inter-day ($n=5$)							
DAB	25	2.32	2.40	0.12	16.30	18.20	20.00
Spermine	25	7.45	7.84	0.47	51.87	43.22	22.28
Double coating	40	0.33	0.36	0.03	17.09	19.53	4.42
Double coating	20	1.38	1.46	0.08	13.39	10.29	5.87

[a] For examples of electropherograms and conditions refer to panels A of Figs. 2 and 5–7.
[b] Percentage area of disialo-Tf relative to the area of the tetrasialo-Tf peak.
[c] Peak area divided by detection time.

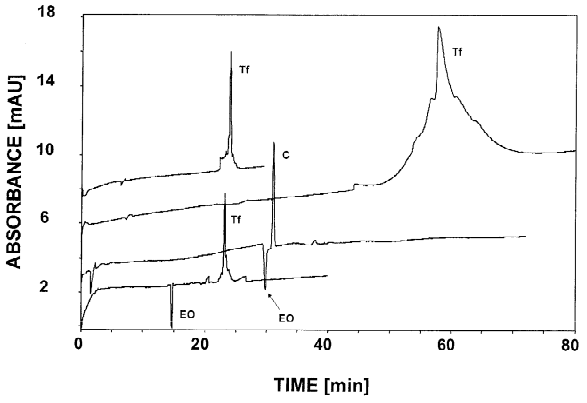

Fig. 3. Electropherograms obtained with Tf standard (0.5 mg/ml in water) using 100 m*M* borate buffers of pH 8.3 with spermine in a capillary of 25 μm I.D. and 50 cm effective length. The spermine concentration varied between 3.0, 0.5, 0.1 and 0.02 m*M* (graphs from top to bottom, respectively). The applied voltages were −20 kV, −20 kV, 20 kV and 20 kV, respectively, and the currents were about 30 μA in all four cases. Injection was effected for 27 s at 0.5 p.s.i. and the cartridge temperature was 25 °C in all cases. For the sake of clarity, electropherograms are presented with *y*-scale offsets. Key as for Fig. 1.

Having a spermine concentration of 0.1 m*M*, normal polarity (detector at the cathode) and otherwise identical experimental conditions as described above, it was possible to detect the EOF within about 30 min ($m_{eo} = 0.84 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}), but Tf did not reach the detector within 70 min (second graph from bottom in Fig. 3). Thus, for analysis of Tf, electroosmosis appears to be to too weak. With 0.05 to 0.02 m*M* spermine, electroosmosis was observed to increase as the spermine concentration was decreased, and almost fully resolved Tf patterns were noted. Best results were obtained with 0.02 m*M* spermine ($m_{eo} = 1.71 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}) and having an effective capillary length of 50 cm (bottom graphs in Figs. 3 and 4). With an injection time of 27 s (pressure: 0.5 p.s.i.), the observed separation characteristics were found to be similar to those obtained with 2.25 m*M* DAB in a fused-silica capillary of the same length and I.D. (Table 1). The analysis time, however, was somewhat longer with spermine. Baseline separation of di- and trisialo-Tf was obtained within 27 min and pentasialo-Tf was not fully separated from tetrasialo-Tf (Table 1). No improvements were noted with shorter capillaries (tested with 30 cm effective length) and with 15 μm I.D. capillaries. However, increased detector signals and an overall better separation was obtained with 50 μm I.D. capillaries of 50 cm total length (Fig. 5, Table 1). As was the case with DAB (Fig. 2), the borate concentration had to be reduced to 50 m*M*. With a voltage of 20 kV, the temperature control of the cartridge being set to 25 °C, an injection time of 4 s (pressure: 0.5 p.s.i.) and a spermine concentration of 0.020 m*M*, Tf with its highly resolved isoforms reached the detector within about 16.0 to 17.5 min (Fig. 5). The concentration of the capillary conditioner was found to be critical. With 0.025 m*M* spermine, all Tf isoforms were detected within 20–26 min, whereas with 0.01 m*M* spermine, incompletely resolved Tf isomers reached the detector within about 12 min. Furthermore, within a set of experiments, a tendency to shorter analysis times was noted using this capillary conditioning agent.

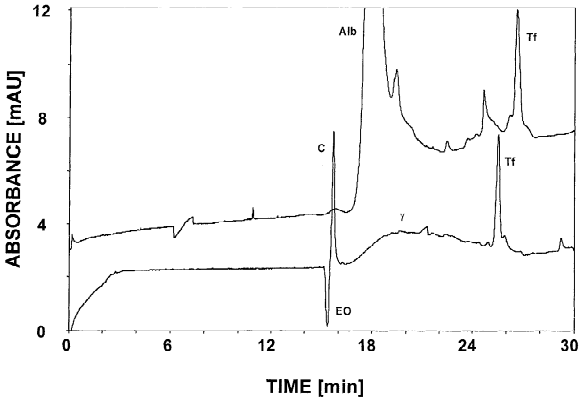

Fig. 4. Electropherograms obtained with a serum of a healthy individual (10-fold diluted after iron saturation) in presence of 3.0 m*M* spermine (upper graph, reversed polarity) and of 0.02 m*M* spermine (lower graph, normal polarity). Other conditions as for top and bottom graphs, respectively, of Fig. 3. Alb refers to albumin.

The data presented in Fig. 5 represent typical serum Tf patterns of a healthy individual (Fig. 5A) and an alcohol abuser (Fig. 5B), samples that were also analyzed with DAB (Fig. 2). The Tf-indices were calculated to be 1.34 and 9.04%, respectively, indicating that the separation performance for di- to pentasialo-Tf was the same as with 3 m*M* DAB (Fig. 2). It is important to note, that asialo-Tf and hexasialo-Tf could not be detected as well (Fig. 5B). The peaks marked with an asterisk are of unknown origin and were present in most electropherograms. Typical

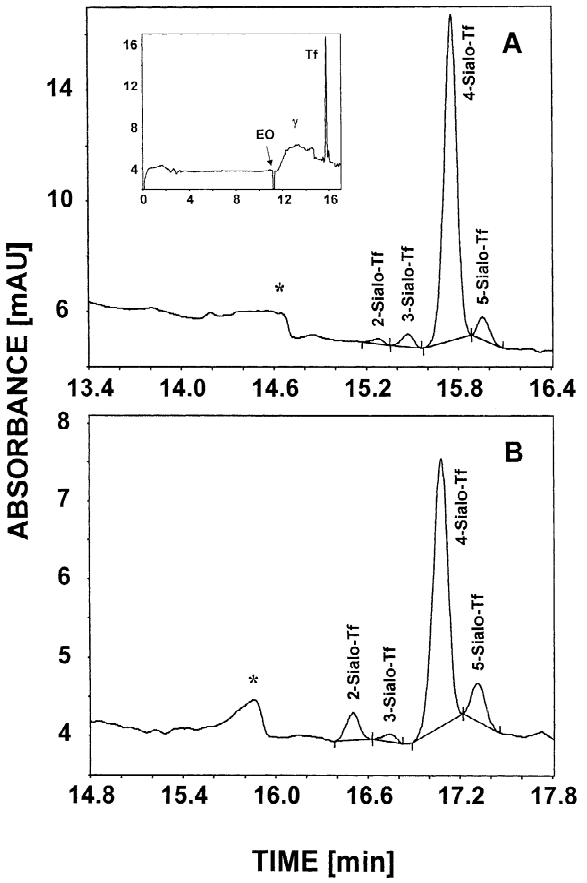

Fig. 5. Electropherograms in presence of 0.02 m*M* spermine obtained with (A) a serum of a healthy individual and (B) a serum of an alcohol abuser in a 50 μm I.D. capillary of 50 cm effective length. The borate buffer concentration was 50 m*M*, the injection time (pressure) was 4 s (0.5 p.s.i.), the voltage was 20 kV and the currents were about 57 μA. The temperature of the cartridge was set to 25 °C. The insert in panel A depicts the entire electropherogram. Key as for Fig. 1.

reproducibility data for peak efficiency, peak shape and resolution obtained via analysis of a serum of a healthy individual are summarized in Table 2, whereas corresponding RSD values for peak detection time, peak area and the Tf-index are presented in Table 3. Compared to the configuration with DAB, imprecision of detection times, relative detection time and peak areas were found to be larger. The RSD of the Tf-index, however, was comparable. Furthermore, it was interesting to find that the electroosmotic mobility and power level for the data of Fig. 5A were almost identical to those of the data with 3 m*M* DAB presented in Fig. 2A ($2.24 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} and 1.9 W/m, respectively).

3.3. Experiments with dynamic double coating

Experiments with the proprietary double coating were primarily performed according to the instructions of the manufacturer of the CEofix CDT-Kit (see Section 2.6). For analysis of a serum of a healthy individual, resolution of Tf isoforms with two to six sialic acid residues was obtained within 7 min (Fig. 6A, Table 1). Additionally, asialo-Tf could be nicely detected in selected sera from alcohol abusers (Fig. 6B). The electroosmotic mobility was calculated to be $3.73 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}, a value that is 66.5% higher compared to those characteristic for the corresponding assays with DAB (Fig. 2) and spermine (Fig. 5). The power level was significantly lower, namely 1.21 W/m compared to 1.9 W/m. The peak height of the main peak (tetrasialo-Tf) was about 40 mAU, i.e. the signal was about 4–6 times higher than the signal of the corresponding peak when analyzed with the assays comprising DAB or spermine as capillary conditioning agents (compare with Figs. 2 and 5).

The data presented in Fig. 6 represent typical serum Tf patterns of a healthy individual (Fig. 6A) and an alcohol abuser (Fig. 6B). The two Tf-indices were calculated to be 1.30 and 20.06%, respectively. Thus, CZE with the double coating was found to correctly classify the two sera. The same was found to be true for the %CDT TIA test that revealed values of 2.0 and 13.6%, respectively. It is important to note the serum of the alcohol abuser whose data are shown in Fig. 6B is not the same as was used to produce the patterns shown in Figs. 2B and 5B. The latter serum lead to a Tf-index of 7.7% and a very small asiolo-Tf peak and was thus not depicted. Typical reproducibility data for peak efficiency, peak shape and resolution obtained via analysis of a serum of a healthy individual are summarized in Table 2, whereas corresponding RSD values for peak detection time, peak area and the Tf-index are presented in Table 3. Compared to the assays with DAB and spermine, RSD values for all parameters were found to be significantly lower. Thus, the double

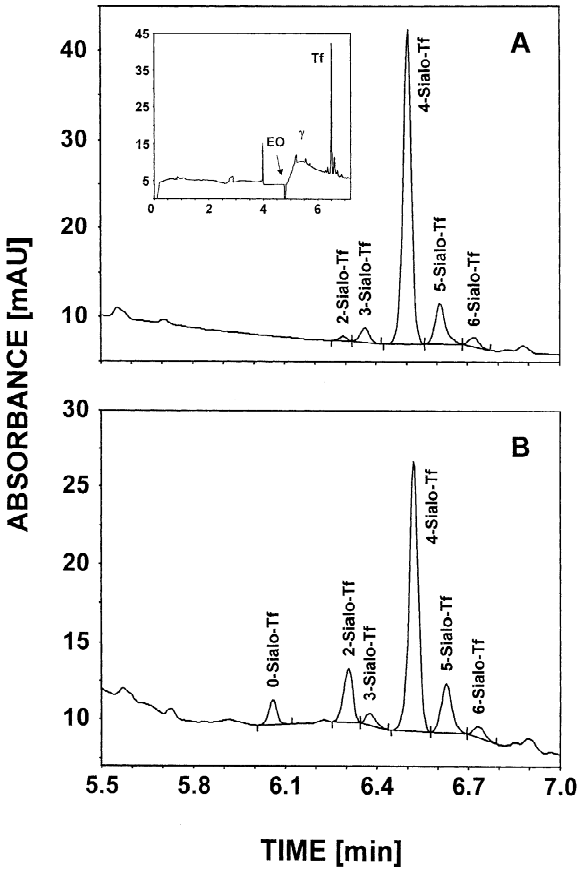

Fig. 6. Electropherograms obtained with the dynamic double coating for analysis of (A) a serum of a healthy individual and (B) a serum of an alcohol abuser having a 50 μm I.D. capillary of 50 cm effective length. The injection time was 10 s (0.3 p.s.i., vacuum), the voltage was 28 kV and the currents were 26.0 μA. The temperature of the cartridge was 40 °C. The insert in panel A depicts the entire electropherogram. Key as for Fig. 1.

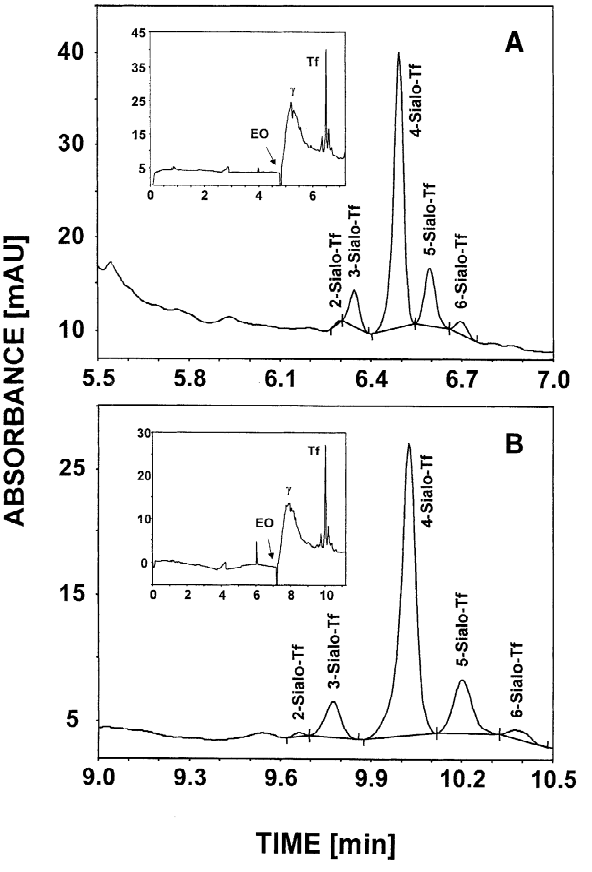

Fig. 7. Electropherograms obtained with a patient serum containing a high amount of trisialo-Tf after analysis with the dynamic double coating at (A) 40 °C and (B) 20 °C and otherwise identical conditions as for Fig. 6. The current for panel B was 15.7 μA. The inserts depict the entire electropherograms. Key as for Fig. 1.

coating appears to abolish the protein wall interactions more efficiently and/or provides improved resurfacing for each analysis.

Although not visible for the data presented in Fig. 6A and with the calculated resolution value given in Table 1, resolution between di- and trisialo-Tf was typically noted to be lower compared to the cases with DAB and spermine (Table 2). For selected patient sera with high amounts of trisialo-Tf (Fig. 7B), this was found to become a problem. For analysis under normal conditions, incomplete separation of di- and trisialo-Tf was noted (Fig. 7A, $R_s = 0.78$, Tf-index $= 0.35\%$). The amount of trisialo-Tf relative to tetrasialo-Tf was determined to be 12.5%, a value that is about 2.5-fold higher compared to the 4.9% determined for the serum of Fig. 6A. Analysis of the same serum with the cartridge temperature reduced to 20 °C (electroosmotic mobility of $2.56 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}, power level of 0.73 W/m) and otherwise identical conditions, revealed a run time of about 10.5 min, a resolution between di- and trisialo-Tf of 1.42, a Tf-index of 0.75% and a relative amount of trisialo-Tf of 12.6% (Fig. 7B). Furthermore, this patient serum was also analyzed in an elongated capillary (70 cm instead of 50 cm effective length) held at 20 °C which resulted in a run time of about 20 min and a Tf-index of 0.82%

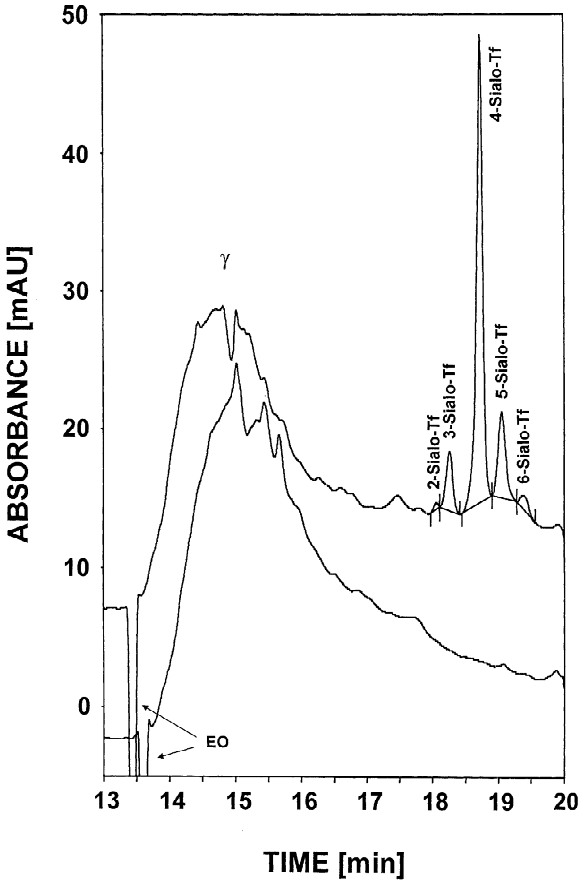

Fig. 8. Electropherograms obtained with a patient serum containing a high amount of trisialo-Tf before (upper graph) and after (lower graph) immunosubtraction for analysis with the dynamic double coating in a 50 μm I.D. capillary of 70 cm effective length and a cartridge temperature of 20 °C. Injection was performed by applying a vacuum of 0.6 p.s.i. for 10 s. The current was 12.1 μA. Other conditions as for Fig. 6. For the sake of presentation, the upper graph is depicted with a 9-mAU *y*-axis shift.

(Fig. 8, upper graph). Using immunosubtraction as described in Section 2.9, all assigned peaks were determined to represent Tf (Fig. 8, lower graph). For comparison of the electropherograms it is important to note that the immunosubtracted sample was monitored with a time shift of about 10 s, an effect that originates from the difference of the sample matrix. Furthermore, misinterpretation of the electropherograms was prevented with incubation of serum with neuraminidase for increasing times and analysis of a range of samples with which the sequential cleavage of the sialic acid moieties could be visualized (data not shown). Elongation of the capillary provided no significant improvement of separation (Table 1). Thus, based upon the resolution increase (Table 2) and the excellent reproducibility (Table 3) obtained for operation at 20 °C, it can be concluded that the use of the 50 cm capillary should be sufficient for monitoring CDT in clinical and forensic practice.

According to the CZE data presented in Figs. 7 and 8, the analyzed serum with increased trisialo-Tf was classified as having a normal Tf-index (<2.27% [25]). However, the %CDT TIA test revealed a CDT value of 3.6% which is considered to be positive according to the reference values proposed in the instruction manual of the test kit (<2.6%) and used in clinical practice (<3.0% [12]). The inaccuracy of the %CDT turbidimetric immunoassay appears to be obvious for such cases. Thus, should a positive immunoassay test result lead to serious consequences, it should always be confirmed by a more specific method, such as CZE [25] or HPLC [12,14].

4. Conclusions

The major problems in analyzing CDT with CZE are the interaction of the macromolecules with the capillary wall and the maintenance of the capillary surface to guarantee reproducibility. As permanently coated capillaries are expensive and were found to deteriorate with time, the use of plain capillaries together with buffer additives that provide a dynamic coating appears to be the most effective approach in terms of both analytical performance and cost. In our work with the P/ACE MDQ instrument, configurations with two amine modifiers, DAB and spermine, and a proprietary commercially available double coating were investigated and found to be suitable for determination of CDT in human serum. For all three approaches, the best electropherograms, in terms of Tf isoform resolution, detection signals and run times, were obtained in 50-μm-I.D. capillaries of 50 cm effective length, application of normal polarity (electroosmosis towards the cathode) and a capillary cartridge temperature of 20–25 °C. Having 3 m*M* DAB or 0.02 m*M* spermine in a borate-based running buffer of pH 8.3 provided data of remark-

able similarity. With these approaches and having an applied power of 1.9 W/m at 20 kV, di-, tri-, tetra- and pentasialo-Tf can be separated and detected within 15–18 min. Asialo-Tf and hexasialo-Tf, however, cannot be monitored. Data evaluated, based upon % area of disialo-Tf in relation to tetrasialo-Tf, were found to be comparable and reproducible. Furthermore, the use of DAB was noted to provide better reproducibility of detection times and higher temperatures lead to reduced run times but insufficient separability in both cases. With the double coating and the buffers provided in the commercial CDT-kit, asialo-Tf and Tf isoforms with two to six sialic acid residues are monitored. The capillary is first rinsed with an initiator (containing a proprietary polycation in a malic acid/arginine buffer at pH 4.8) followed by introduction of the running buffer (containing a proprietary polyanion in a Tris/boric acid buffer at pH 8.5) and analysis of the iron saturated sample. Using the recommended cartridge temperature of 40 °C, excellent reproducibility and short run times of about 7 min were noted. Compared to the assays based upon DAB and spermine, signal magnitudes were determined to be significantly higher and the applied power level lower (1.21 W/m at 28 kV). The resolution of di- and trisialo-Tf, however, was incomplete with sera containing high amounts of trisialo-Tf. To obtain complete Tf isoform resolution for these cases, the cartridge temperature had to be lowered to 20 °C which provided run times of about 10 min (power level of 0.73 W/m) and excellent reproducibility. Based upon the evaluations reported in this paper, the use of the double coating at 20 °C together with the commercially available CEofix-CDT-kit currently appears to be the best approach for CZE analysis of CDT in a routine arena.

Acknowledgements

The authors acknowledge the receipt of the Analis CEofix CDT kits from its manufacturer (Analis, Namur, Belgium). This work was supported by the Liver Foundation, Berne, Switzerland, the Swiss National Science Foundation and MIUR (Italian Ministry of Education, University and Research, project 2001065132).

References

[1] T. Arndt, Clin. Chem. 47 (2001) 13.
[2] F. Tagliaro, F. Bortolotti, F. Crivellente, F. Cittadini, Forensic Sci. Rev. 12 (2000) 134.
[3] H. Stibler, K.G. Kjellin, J. Neurol. Sci. 30 (1976) 269.
[4] H. Stibler, C. Allgulander, S. Borg, K.G. Kjellin, Acta Med. Scand. 204 (1978) 49.
[5] H. Stibler, S. Borg, C. Allgulander, Acta Med. Scand. 206 (1979) 275.
[6] P. Sillanaukee, N. Strid, J.P. Allen, R.Z. Litten, Alcohol. Clin. Exp. Res. 25 (2001) 34.
[7] G. de Jong, H.G. van Ejik, Electrophoresis 9 (1988) 589.
[8] E. Landberg, P. Påhlsson, A. Lundblad, A. Arnetorp, J.-O. Jeppsson, Biochem. Biophys. Res. Commun. 210 (1995) 267.
[9] T. Inoue, M. Yamauchi, K. Ohkawa, Electrophoresis 20 (1999) 452.
[10] J.-O. Jeppsson, H. Kristensson, C. Fimiani, Clin. Chem. 39 (1993) 2115.
[11] E. Werle, G.E. Seitz, B. Kohl, W. Fiehn, H.K. Seitz, Alcohol Alcohol. 32 (1997) 71.
[12] A. Helander, M. Fors, B. Zakrisson, Alcohol Alcohol. 36 (2001) 406.
[13] M. Lipowski, L. Dibbelt, M. Seyfarth, Clin. Biochem. 33 (2000) 635.
[14] U. Turpeinen, T. Methuen, H. Alfthan, K. Laitinen, M. Salaspuro, U.-H. Stenman, Clin. Chem. 47 (2001) 1782.
[15] S. Molteni, W. Thormann, J. Chromatogr. 638 (1993) 187.
[16] F. Kilàr, S. Hjertén, Electrophoresis 10 (1989) 23.
[17] F. Kilàr, S. Hjertén, J. Chromatogr. 480 (1989) 351.
[18] R.P. Oda, J.P. Landers, Electrophoresis 17 (1996) 431.
[19] L. Yang, Q. Tang, A.K. Harrata, C.S. Lee, Anal. Biochem. 243 (1996) 140.
[20] O. Iourin, T.S. Mattu, N. Mian, G. Keir, B. Winchester, R.A. Dwek, P.M. Rudd, Glycoconj. J. 13 (1996) 1031.
[21] R. Prasad, R.L. Stout, D. Coffin, J. Smith, Electrophoresis 18 (1997) 1814.
[22] R.P. Oda, R. Prasad, R.L. Stout, D. Coffin, W.P. Patton, D.L. Kraft, J.F. O'Brien, J.P. Landers, Electrophoresis 18 (1997) 1819.
[23] F. Tagliaro, F. Crivellente, G. Manetto, I. Puppi, Z. Deyl, M. Marigo, Electrophoresis 19 (1998) 3033.
[24] F. Crivellente, G. Fracasso, R. Valentini, G. Manetto, A.P. Riviera, F. Tagliaro, J. Chromatogr. B 739 (2000) 81.
[25] F. Tagliaro, F. Bortolotti, M. Zuliani, F. Crivellente, G. Manetto, V.L. Pascali, M. Marigo, J. Cap. Electrophoresis 6 (1999) 137.
[26] B.C. Giordano, M. Muza, A. Trout, J.P. Landers, J. Chromatogr. B 742 (2000) 79.
[27] A.L. Trout, R. Prasad, D. Coffin, A. DiMartini, T. Lane, C. Blessum, N. Khatter, J.P. Landers, Electrophoresis 21 (2000) 2376.
[28] A.T. Beisler, R.H. Kelly, J.P. Landers, Anal. Biochem. 285 (2000) 143.
[29] B. Wuyts, J.R. Delanghe, I. Kasvosve, A. Wauters, H. Neels, J. Janssens, Clin. Chem. 47 (2001) 247.

[30] H. Katayama, Y. Ishihama, N. Asakawa, Anal. Chem. 70 (1998) 2254.
[31] F. Tagliaro, F. Bortolotti, R.M. Dorizzi, M. Marigo, Clin. Chem. 48 (2002) 208.
[32] P.G. Righetti, C. Gelfi, B. Verzola, L. Castelletti, Electrophoresis 22 (2001) 603.
[33] J. Horvath, V. Dolník, Electrophoresis 22 (2001) 644.
[34] B. Verzola, C. Gelfi, P.G. Righetti, J. Chromatogr. A 868 (2000) 85.
[35] M.E. Legaz, M.M. Pedrosa, J. Chromatogr. A 719 (1996) 159.
[36] M.E. Lacey, A.G. Webb, J.V. Sweedler, Anal. Chem. 72 (2000) 4991.