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Improved method for carbohydrate-deficient transferrin determination in human serum by capillary zone electrophoresis

Federica Crivellente[a], Giulio Fracasso[b], Roberta Valentini[a], Giulia Manetto[a], Anna Pia Riviera[b], Franco Tagliaro[a,c,*]

[a]*Institute of Forensic Medicine, University of Verona, Verona, Italy*
[b]*Institute of Immunology and Infectious Diseases, University of Verona, Verona, Italy*
[c]*Institute of Forensic Medicine, Catholic University of the Sacred Heart, Rome, Italy*

Abstract

Carbohydrate-deficient transferrin (CDT) is a reliable marker of chronic or repeated alcohol abuse. It indicates a group of isoforms of human transferrin (Tf), the main iron transport serum protein, deficient in sialic acid residues (asialo-, monosialo- and disialo-Tf) in comparison to the main isotransferrin which contains four sialic acid groups (tetrasialo-Tf). The aim of the present work was to develop a capillary electrophoretic method suitable for rapid determination of CDT components in serum. Serum samples (0.1 ml) were saturated with iron by incubation with 10 m*M* $FeCl_3$ (2 μl) and 500 m*M* $NaHCO_3$ (3 μl) for 30 min, then diluted 1:10 in water and injected by positive pressure (0.5 p.s.i. for 10 s). Separation was performed with a capillary zone electrophoretic method using bare fused-silica capillaries (57 cm×20 μm I.D.) and a buffer composed of 100 m*M* sodium tetraborate adjusted with 6 *M* HCl to pH 8.3 added with 1.5 m*M* diaminobutane. Applied voltage was 20 kV and temperature 25°C. Detection was by UV absorption at 200 nm wavelength. Under the described conditions, asialo-, monosialo-, disialo-, trisialo- and tetrasialo-transferrin were baseline separated. The limit of detection (signal-to-noise ratio of 2) was about 0.3% for disialo-Tf, and 0.5% of trisialo-Tf, expressed as percentages of the terasialo-Tf peak area. Day-to-day RSDs of relative migration times were ≤0.2%. Quantitation showed day-to-day RDSs ≤6.9% and ≤10.9% for disialo- and trisialo-Tf, respectively. The results from 79 control subjects, including social drinkers, and 23 alcoholics showed disialo- and trisialo-Tf significantly increased in patients ($P<0.0001$ and <0.01, respectively). A clear interference from trisialo-Tf in an immunoassay for CDT was demonstrated. The present method is suitable for confirmation of CDT immunoassays by independent technique.

Keywords: Transferrins; Carbohydrate-deficient transferrin

1. Introduction

Among the different markers of alcohol abuse [1] including ethanol metabolites and congeners, enzymes (GGT, AST, ALT), acetaldehyde adducts, high-density lipoprotein cholesterol, 5-hydroxytryptophol and 5-hydroxytryptophol-3-acetic acid, dolichols etc., carbohydrate-deficient transferrin (CDT), introduced at the end of the 1970s by Stibler et al., has been extensively studied [2,3] and is nowadays considered the most reliable marker for continuous excessive alcohol consumption [3].

*Corresponding author. Corresponding address: Institute of Forensic Medicine, University of Verona, Policlinico Borgo Roma, 37134 Verona, Italy. Tel.: +390-45-8074-618; fax: +390-45-505-259.
E-mail address: ftmedl@borgoroma.univr.it (F. Tagliaro)

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CDT is related to a microheterogeneity of serum transferrin (Tf), the major iron transporting glycoprotein, which may contain from zero up to eight sialic acid residues. The major Tf isoform (>90%) in the serum of human subjects contains four sialic acid residues (tetrasialo-Tf) (after complete Fe^{3+} saturation, isoelectric point, pI=5.4 [2]). As a result of alcohol abuse, some less glycosilated isoforms with pI values of 5.7 (disialo-Tf) and 5.9 (asialo-Tf) are reported to increase and are collectively named CDT [2]. The exact mechanism of this phenomenon has not been clarified yet, but the inhibition of glycosylation enzymes mediated by acetaldehyde, the major metabolite of ethanol, is probably involved.

On the basis of a large body of literature, it is believed that an ethanol intake >50–80 g/day for one to two weeks leads to abnormal increase of serum CDT, which has a half-life of about 15 days [3]. The diagnostic specificity of CDT, a crucial parameter for application in the forensic environment, is reported to be good-to-excellent (90–100%). Indeed, increases of CDT not related to alcohol abuse have been observed only in pregnancy, biliary hepatic cirrhosis and carbohydrate deficient glycoprotein syndrome (CDG syndrome) [2,4–6].

Until recently, the analytical determination of CDT has been based on isoelectric focusing (IEF) combined with immunofixation, zone immunoelectrophoresis or Western blotting or on chromatographic techniques (i.e., anion-exchange chromatography or chromatofocusing) followed by immunoassays. These mutistep techniques are clearly too complex and time consuming for routine application and suffer from poor reliability, due to the complicated and manual procedures.

Nowadays, CDT assay is routinely carried out in many laboratories using commercial kits based on a two-step procedure, including removal of trisialo- and more glycosilated Tf's by ion-exchange microcolumns and determination of the remaining Tf-like immunoreactivity (expressing CDT) by immunoassay [7,8]. However, to meet the basic requirements for any analysis potentially carried out for forensic and/or administrative purposes, as often CDT assay is, results from immunoassays need to be confirmed by an independent, non correlated analytical method (e.g., chromatography or electrophoresis).

Since in everybody's experience the quantitative reliability of gel electrophoresis or chromatography off-line coupled to immunoassay is generally poor, to the best of our knowledge, the only real alternative is represented by high-performance liquid chromatography (HPLC) with visible radiation absorbance detection (460 nm) [9]. In fact, HPLC–UV–Vis is based physico-chemical characteristics of the molecules which are independent of ''antigenicity'' and, therefore, can be considered inherently ''orthogonal'' to immunoassays. Unfortunately, HPLC–UV–Vis [9], even in its more recent evolution [8,21], needs complex sample preparation, column regeneration and gradient elution. Moreover, the chromatographic columns are very expensive and delicate.

Already in 1989, Kilar and Hjertén reported the successful application of capillary electrophoresis (CE) in either capillary isoelectric focusing (cIEF) [10] or capillary zone electrophoresis (CZE) [11] modes to resolve the major forms of human Tf (in pure solutions), according to the different content of sialic acid and different degree of iron complexation.

However, in 1996, Oda and Landers [12] published a detailed study aimed at testing the ability of CZE to separate Tf isoforms of different species, including human, with the ultimate goal of developing a clinical diagnostic test for alcoholism and carbohydrate-deficient glycoprotein syndrome. CZE was carried out in uncoated silica capillaries with borate buffers and different cationic additives using UV detection at 200 nm. The results showed the separation of the iron-saturated human Tf isoforms from hexasialo- to trisialo-Tf, but, according to the authors themselves, the assay was inadequate for application to human sera, because of long analysis time and mediocre peak resolution.

Later, Oda et al. [13] and Prasad et al. [14] using DB-17 coated capillaries and buffers containing hydroxy-ethyl cellulose or methyl cellulose, respectively, improved resolution and analysis time. However, to achieve the needed sensitivity for clinical application (i.e., carbohydrate-deficient glycoprotein syndrome and alcohol abuse diagnosis) a sophisticated, expensive and time-consuming sample preparation based on immunoextraction of Tf from serum samples was required before CZE.

On these grounds, we have developed and recently

reported [15] a simple CZE method using 100 m*M* borate buffer, pH 8.3 and uncoated capillaries (20 μm I.D.) with UV detection at 200 nm. This method proved able to separate and determine disialo- and trisialo-Tf isoforms, i.e., the Tf glycoforms of major clinical relevance in human serum, within a reasonable time (20 min) and without the need of a complex sample pre-treatment (limited just to iron saturation and 1:10 sample dilution in water).

Later, this methodology has been improved by addition of a cationic additive to reduce the interactions of the proteins with the capillary wall and by using a longer capillary.

The accuracy of the improved method has been verified by immunosubtraction and immunoextraction from human serum and the procedure has therefore been completely revalidated. This method has then been applied to real cases and compared with a CDT immunoassay in critical samples.

2. Materials and methods

2.1. Standards and chemicals

Holotransferrin (iron saturated, human) approx. 98% pure and neuraminidase (type X from *Clostridium perfrigens*) were purchased from Sigma (St. Louis, MO, USA). Rabbit immunoglobulins to human Tf were obtained from Dakopatts (Glostrup, Denmark). Water of HPLC-grade and other chemicals of analytical grade were from Carlo Erba (Milan, Italy). Diaminobutane (DAB) was obtained from Sigma. A stock solution was prepared by diluting DAB in water to obtain a 100 m*M* solution, which was stored at +4°C.

Radioimmunoassays for CDT were carried out using commercial kits (CDTect, Pharmacia, Uppsala, Sweden) following the manufacturer's instructions.

A Tf-specific immunosorbent was produced by coupling rabbit immunoglobulins from Dakopatts (without further purification) with CNBr-activated Sepharose 4B (Pharmacia). In this operation, the procedure suggested by the manufacturer of the immunoaffinity solid phase was followed, starting with 1 g of solid phase (giving about 3.5 ml of swelled resin) and 1 ml of immunoglobulins. In short, the ligand was dissolved in 0.1 *M* $NaHCO_3$, pH 8.3 containing 0.5 *M* NaCl and mixed with the gel (previously washed with 1 m*M* HCl). The mixture was rotated end-over-end for 2 h at room temperature, then excess ligand was washed away with the coupling buffer and remaining active groups were blocked with 0.1 *M* Tris–HCl, pH 8.0. The product was washed for three cycles with acetate buffer (0.1 *M*, pH 4.0) followed by Tris buffer (0.1 *M*, pH 8.0), both containing NaCl (0.5 *M*) and stored at +4°C until use.

Disposable ultrafiltration tubes (Microconcentrator 30, Amicon, Beverly, MA, USA) were used to desalt and concentrate the eluates from immunoextractions.

2.2. Instrumentation

Experiments were carried with a P/ACE 5500 automated electropherograph (Beckman, Fullerton, CA, USA) with a single-wavelength UV detector with interference filters at 200, 214, 230, 254 and 280 nm. The distance between the detection window and the end of the capillary was 7 cm.

Uncoated fused-silica capillaries were furnished by Beckman. New capillaries were washed with 1 *M* sodium hydroxide for 10 min followed by 0.1 *M* sodium hydroxide for 10 min, water for 10 min and finally conditioned with running buffer for 20 min.

Every day the capillary was washed with 1 *M* NaOH for 5 min, followed by 0.1 *M* sodium hydroxide for 5 min, water for 10 min and running buffer for 15 min. Finally, the system was conditioned by applying 20 kV voltage for about 20 min, until a steady baseline was obtained.

2.3. Electrophoretic conditions

Injection was accomplished by application of a pressure of 0.5 p.s.i. for 10 s (1 p.s.i.=6894.76 Pa). The running buffer was composed of 100 m*M* sodium tetraborate adjusted to pH 8.3 with 6 *M* HCl and added with DAB at a concentration of 1.5 m*M*. The addition of DAB at the used concentration did not alter significantly the pH of the buffer. Other analytical conditions were as follows. Capillary: bare fused-silica 57 cm (50 cm to the detector)×20 μm I.D.; voltage: 20 kV; current: about 20 μA; temperature: 25°C; UV detection: 200 nm wavelength.

2.4. Subjects and sample preparation

Control serum samples were collected from 79 blood donors (35 males and 44 females; aged from 20 to 60 years) who showed no clinical evidence of chronic alcohol abuse (social drinkers were included) and serum transaminases, γ-glutamyltransferase and mean corpuscolar volume (MCV) within the normal ranges. ''Positive'' serum samples were collected from 23 subjects (21 males, two females; aged from 20 to 60 years) with clear anamnestic, clinical and laboratory evidence of chronic alcohol abuse (>50–80 g of ethanol per day), who had just been admitted to an alcohol detoxication program.

In order to avoid dishomogeneity in the charge of the individual Tf glycoforms due to incomplete iron saturation, serum samples (0.1 ml) were saturated with iron (according to Ref. [9]) by incubation with 10 m*M* $FeCl_3$ (2 μl) and 500 m*M* $NaHCO_3$ (3 μl) for 30 min, then diluted 1:10 in water and injected.

2.5. Quantitation, statistical analysis and separation parameters

The quantitation of the peaks corresponding to carbohydrate deficient Tf isoforms was done using their relative areas to the tetrasialo-Tf peak.

Statistical analysis was carried out using descriptive parametric statistics and, for comparisons, the Student *t*-test.

Efficiency, expressed as number of theoretical plates (N), was calculated as: $N=5.54(t/W_{1/2})^2$, where t is the migration time, and $W_{1/2}$ is the peak width at half peak height.

Resolution (R_s) was calculated on the basis of the equation: $R_s=(t_2-t_1)/W_2$, where t_1 and t_2 represent the migration times of two adjacent peaks, W_2 the width at the baseline of the slower moving peak.

2.6. Neuraminidase treatment

Neuraminidase, 1 U/ml in 50 m*M* sodium acetate buffer, pH 5, was used to split off the sialic acid residues from Tf. A 45-μl volume of enzyme solution was incubated with 5 μl of pure Tf solution or human serum at room temperature. The reaction was followed at different times with repeated direct injections of the mixture, until the appearance of the peak attributed to asialo-Tf. The partial hydrolysis products containing asialo-, monosialo-, disialo-, trisialo- and tetrasialo-Tf were used as standard mixtures of Tf isoforms.

2.7. Immunosubtraction

Immunosubtraction of Tf from human serum was carried out as follows: 60 μl of anti-human Tf immunoglobulins (titre 750 μg/ml) was incubated with 25 μl of human serum at +4°C overnight. After centrifugation at 2000 *g* for 20 min at 4°C, the supernatant was collected, diluted 1:3 and injected.

2.8. Immunoextraction

A 1-ml volume of immunosorbent was poured into a void polypropylene column (10 ml) with a glass microfibre filter at the bottom. Human serum (400 μl) was mixed with 1200 μl of a solution composed of 10 m*M* sodium citrate and 25 m*M* disodium phosphate, pH 7.2 (binding buffer) and added to the immunosorbent. The plastic column was sealed and rotated for 2 h at room temperature. Then, the column inlet and outlet were opened and the solid phase was washed with 30 ml of binding buffer; immunoreactive Tf-like material was eluted with 4 ml of pH 2.9 elution buffer containing 10 m*M* sodium citrate and 25 m*M* monosodium phosphate. The eluate was dialysed overnight against 2.5 l of 50 m*M* disodium phosphate, pH 7.4 and finally concentrated to 400 μl with a Microcon 30 microconcentrator. The resulting solution was injected.

3. Results and discussion

3.1. Separation

In the proposed analytical approach, the separation of the Tf isoforms is carried out on the basis of their different mobility, related to the different number of sialic acid groups, each bearing a negative charge. Due to the ''normal'' polarity used (with the anode at the injection end of the capillary) and to the high electroosmotic flow generated at pH 8.3 and directed towards the cathodic end, all Tf isoforms are drawn towards the detector, notwithstanding their negative

charge. Hence, the backward electrophoretic mobility of the analytes causes the less negatively charged isoforms to reach the detector window faster than the isoforms bearing more sialic acid residues (Fig. 1).

The ability of CZE in tiny (20 μm I.D.) bare fused-silica capillaries to separate and determine the carbohydrate deficient isoforms of Tf in human serum has already been demonstrated in a previous paper by our group [15].

In the present work, in order to reduce protein adsorption onto the fused-silica capillary walls, a cationic additive (DAB) was added to the same running buffer (100 m*M* borate, pH 8.3) used in our previous work [15]. This produced a relevant increase in separation efficiency (from about 500 000 to about 1 000 000 theoretical plates per meter). Besides, the increase of capillary length (from 37 to 57 cm) improved the resolution of disialo-, trisialo- and tetrasialo-Tf. This is particularly important in real samples where, to allow the detection of the minor Tf isoforms corresponding to CDT, tetrasialo-Tf (about 95% of all the Tf isoforms) has to be overloaded, giving a broadened peak tending to overlap on trisialo-Tf.

Despite the increase in length, compensated by an increase of electric field (from 270 to 350 V/cm), the analysis time remained acceptable, increasing from about 12 min to about 15 min.

DAB was previously tested in CDT separation by Oda and Landers [12], who reported good results with bovine Tf, but poor results with Tf from other species, including human. Decamethonium bromide was chosen by these authors as the best additive for the separation of human Tf.

On the contrary, in our experience, the latter additive, although effective with pure Tf, gave some spurious peaks in the region of CDT isoforms, when serum samples were injected. On the contrary, DAB allowed a stable and ''clean'' baseline in the crucial region corresponding to the carbohydrate deficient isoforms of human Tf.

The alternative approach to CDT analysis based onto coated capillaries, recently proposed by Oda et al. [13] and Prasad et al. [14], needed the addition of

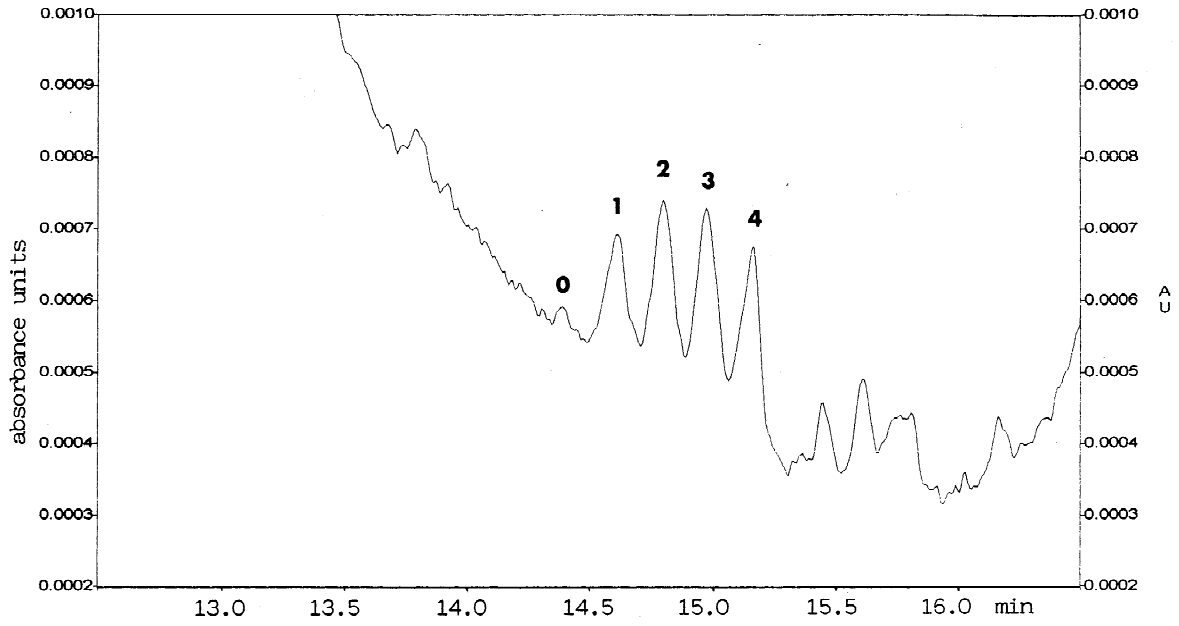

Fig. 1. Electropherogram of the products a partial cleavage of sialic acid residues from serum transferrin by incubation with neuraminidase for 45 min at room temperature. For analytical conditions, see Section 2.3. Peaks: 0=asialo-Tf, 1=monosialo-Tf, 2=disialo-Tf, 3=trisialo-Tf, 4=tetrasialo-Tf.

alkylated celluloses. In our experience, this caused a high background absorbance in the low UV wavelengths with unstable baseline, limiting the analytical sensitivity, which is crucial to determine CDT in serum. Thus, a tedious and time-consuming sample pretreatment including immunoextraction and concentration of CDT from serum was required.

Apparently, the proposed method takes also advantage of the well known complexing activity of borate ions on saccharide groups to achieve the separation of Tf glycoforms. In fact, borate buffers, in our experience, gave constantly better resolution than Tris and phosphate buffers.

3.2. Peak identification

The application of the method to unextracted human serum allowed the identification of the Tf isoforms, not only on the basis of the mere correspondence of migration time with that of standard Tf, but also by immunosubtraction (Fig. 2). A further confirmation came from immunoextraction experiments, in which the different isoforms of Tf-like material recovered from the immunosorbent showed migration times and relative peak areas exactly matching those observed in the direct injection of serum. Figs. 3 and 4 show comparisons between injection of unextracted sera from a control subject and from a subject with high trisialo-Tf and the respective immunoextracted samples. A close correspondence of the CDT peaks is evident.

3.3. Linearity

The linearity of the assay (calculated on tetrasialo-Tf) was good in the range of concentrations from 12.5 μg/ml to 900 μg/ml and is described by the equation $y=0.2068x-0.8905$, $R^2=0.9966$ (where x=concentration in μg/ml and y=peak area).

3.4. Sensitivity

Under the described CZE conditions, the absolute limit of detection (LOD) of human Tf (signal-to-noise ratio of 2) was about 6 μg/ml. The LOD of the carbohydrate deficient Tf isoforms, as percentages of the tetrasialo-Tf peak area, was 0.3% for disialo-Tf and 0.5% for trisialo-Tf. Thus, the sensitivity was perfectly adequate for the purpose of determining disialo- and trisialo-Tf in human serum, also according to the percentage concentrations reported by Martensson et al. [16] corresponding to about 2% and 6% of human Tf, respectively.

Unfortunately, we could not determine asialo-Tf in serum, which is also reported to be correlated with alcohol use. Indeed, its concentration is as low as 0.3% of Tf [16] and therefore too close to the sensitivity limit to allow its determination.

The minor Tf isoforms with higher degree of glycosylation than tetrasialo-Tf were not included in the present study, because of their minor relevance as markers of alcohol abuse.

3.5. Precision

Analytical precision was investigated by calculating intra-day and day-to-day reproducibility of migration times and peak areas of disialo-, trisialo- and tetrasialo-Tf from a normal and a pathological serum (CDTect=12 and 40 U/l, respectively). The results of the precision tests are shown in Tables 1–4.

The precision of absolute migration times was good, with day-to-day relative standard deviations (RSDs)≤1.8%, but much better was that of relative values, based on the migration time of tetrasialo-Tf, which was characterised by RSDs≤0.2%.

3.6. Quantitative analysis

CDT quantitation was expressed as area % of the tetrasialo-Tf peak, in order to compensate for variations in serum whole Tf concentrations. Disialo- and trisialo-Tf showed acceptable quantitative reproducibility in both intra-day and day-to-day experiments (see Tables 3 and 4).

Because of the lack of pure standards of the Tf glycoforms and/or certified sera with known CDT content, it was not possible to verify directly the analytical accuracy. Moreover, no ‘‘reference’’ quantitative analytical methods for the determination of the Tf isoforms are nowadays easily accessible, where IEF is inherently imprecise in quantitation and HPLC is available only in few specialised laboratories.

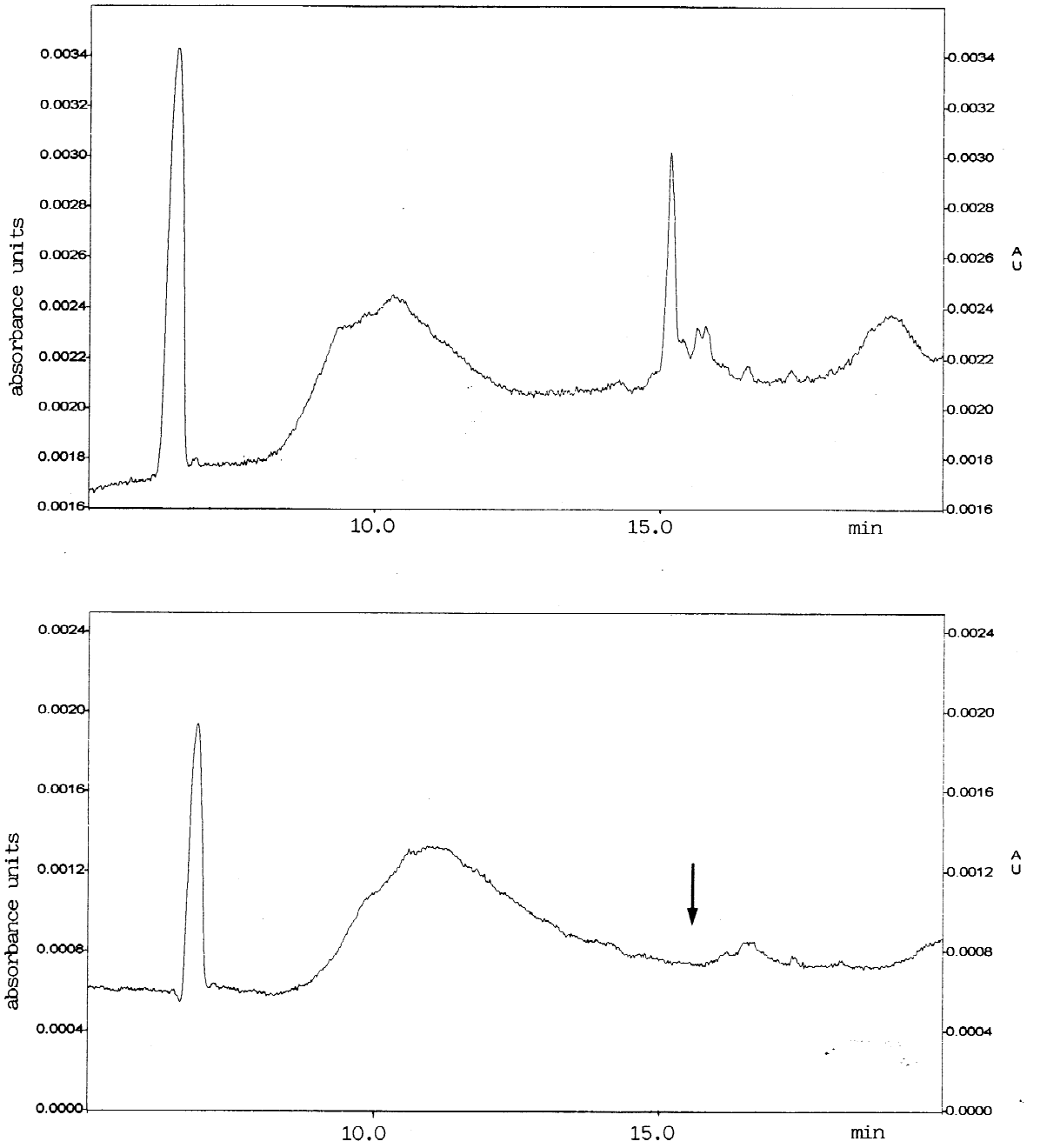

Fig. 2. Immunosubtraction experiment of Tf from human normal serum. For method, see Section 2.7. Upper electropherogram: normal serum before immunosubstraction; lower electropherogram: the same serum sample after immunosubtraction. The arrows indicate Tf migration time.

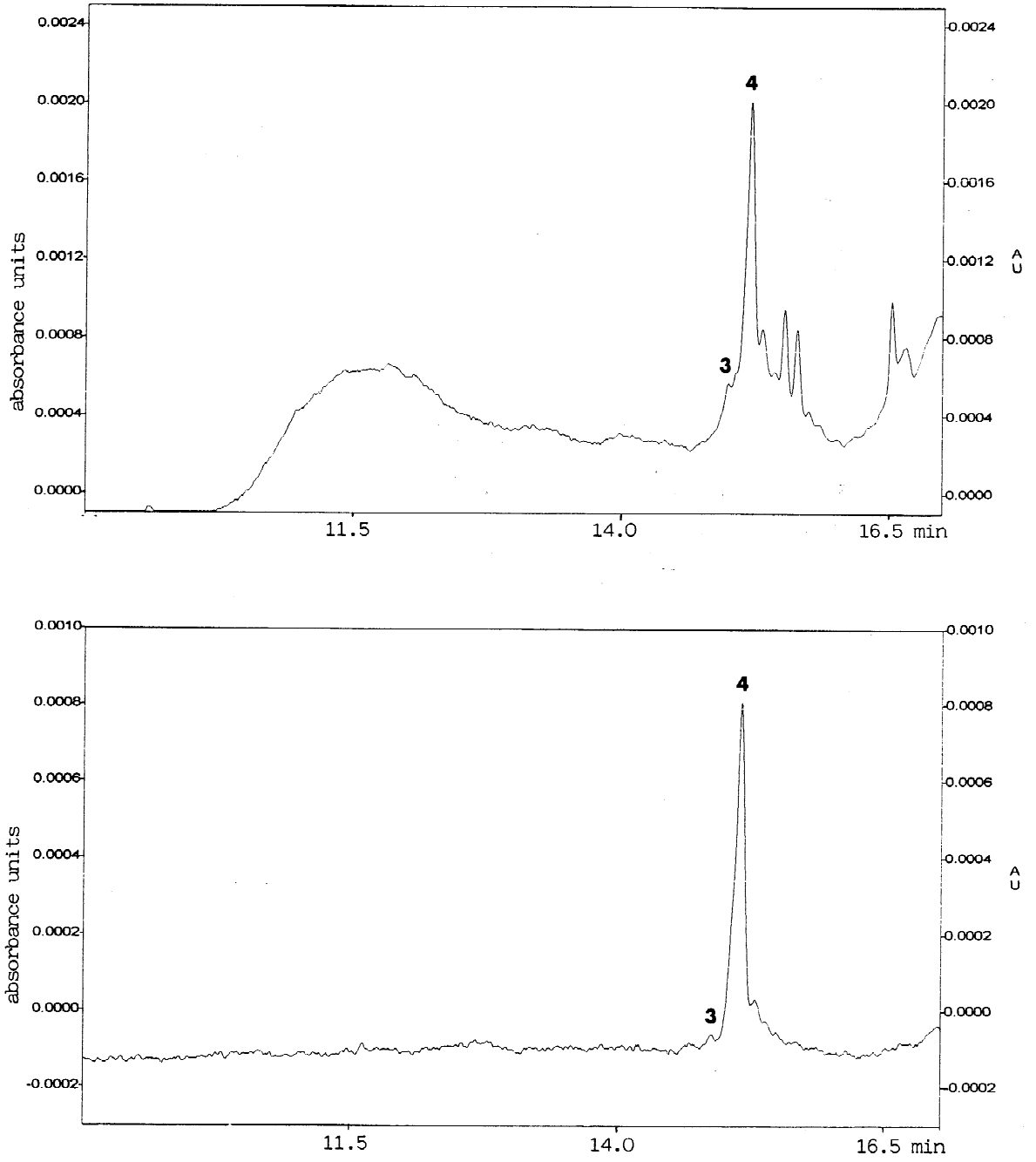

Fig. 3. Immunoextraction experiment of Tf from human normal serum. For method, see Section 2.8. Upper electropherogram: serum sample before immunoextraction; lower electropherogram: immunoextracted material from the same sample.

However, immunoextraction of sera gave an excellent concordance with the direct determination and no analytical interferences were found in subjects suffering from the most common chronic illnesses (aterosclerosis, coronary insufficiency, hypertension, hepatic diseases etc.)

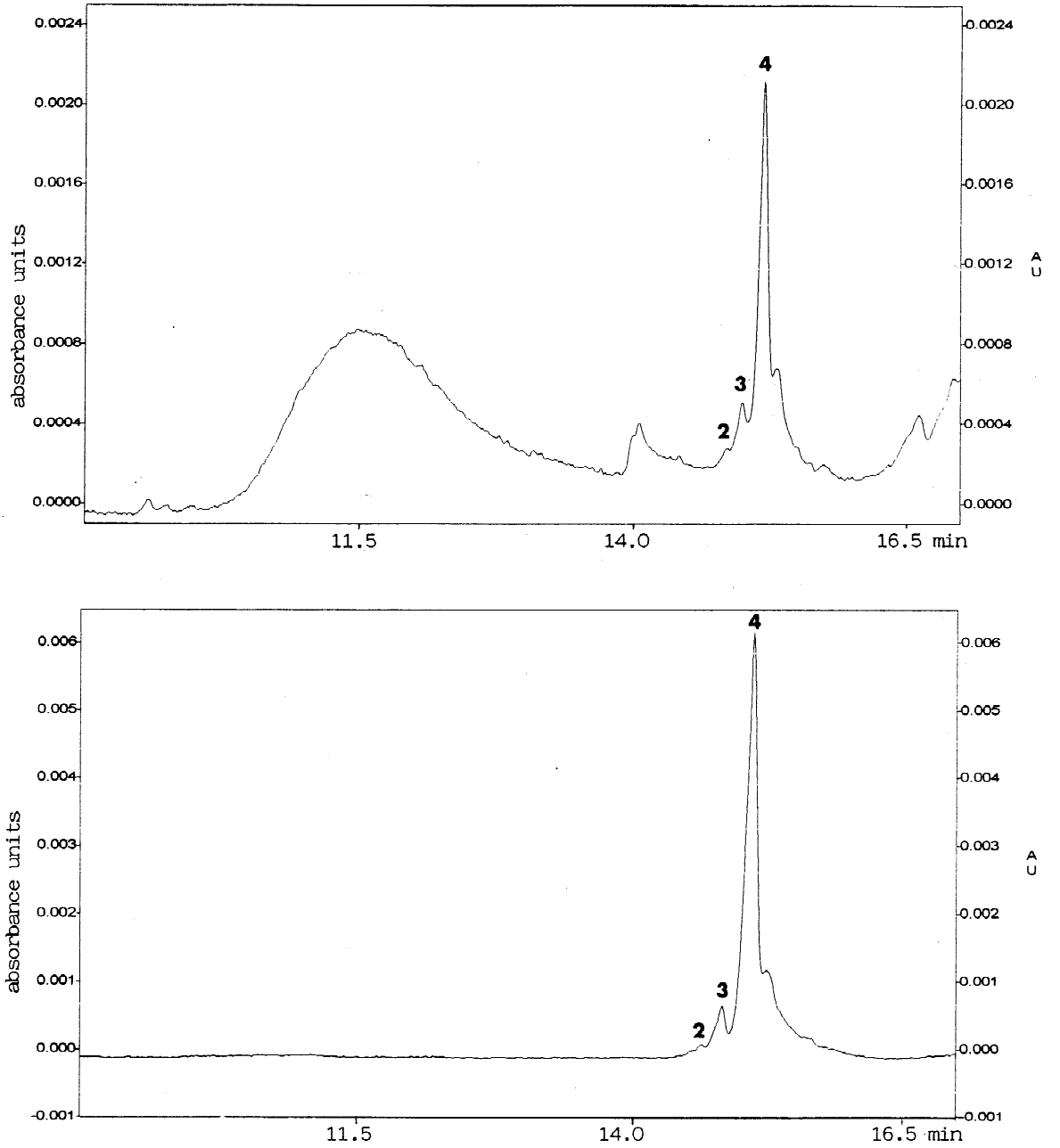

Fig. 4. Immunoextraction experiment of Tf from human serum with high trisialo-Tf. For method, see Section 2.8. Upper electropherogram: serum sample before immunoextraction; lower electropherogram: immunoextracted material from the same sample.

3.7. Clinical results

Fig. 5 shows a comparison between the electropherogram from a control subject (CDTect=10 U/l) and that from an alcohol abuser (CDTect=97 U/l): a neat difference in the peak corresponding to disialo-Tf, the major component of CDT, is evident. In the latter sample, a peak with a migration time

Table 1
Repeatability of the CZE determination of disialo-, trisialo- and tetrasialo-Tf, calculated on a control serum (CDTect=12 U/l) (absolute values)

	Intra-day (n=6)		Day-to-day (n=5)	
	Mean	RSD (%)	Mean	RSD (%)
Migration time (min)				
Disialo-Tf	14.3	0.5	14.5	1.7
Trisialo-Tf	14.5	0.5	14.7	1.7
Tetrasialo-Tf	14.7	0.5	14.9	1.7
Peak area[a]				
Disialo-Tf	112.7	8.7	110.3	9.7
Trisialo-Tf	221.0	10.1	225.2	13.7
Tetrasialo-Tf	16 568.6	5.4	16 864.3	6.1

[a] Arbitrary units.

Table 2
Repeatability of the CZE determination of disialo-, trisialo- and tetrasialo-Tf, calculated on a serum from an alcoholic (CDTect=40 U/l) (absolute values)

	Intra-day (n=6)		Day-to-day (n=5)	
	Mean	RSD (%)	Mean	RSD (%)
Migration time (min)				
Disialo-Tf	14.2	0.2	14.4	1.8
Trisialo-Tf	14.5	0.3	14.6	1.8
Tetrasialo-Tf	14.6	0.3	14.8	1.7
Peak area[a]				
Disialo-Tf	690.4	8.9	700.7	10.1
Trisialo-Tf	557.0	9.1	567.5	10.7
Tetrasialo-Tf	9300.0	8.9	9397.3	9.1

[a] Arbitrary units.

corresponding to asialo-Tf was also identified, but no further confirmation was performed.

The results from analysis of 79 sera from control subjects, including social drinkers, and 23 alcoholics are summarised in Table 5.

In brief, control males showed disialo-Tf per-

Table 3
Repeatability of the CZE determination of disialo- and trisialo-Tf in the control serum as relative values, calculated on the respective tetrasialo-Tf peak

	Intra-day (n=6)		Day-to-day (n=5)	
	Mean	RSD (%)	Mean	RSD (%)
Migration time				
Disialo-Tf	0.972	0.1	0.970	0.2
Trisialo-Tf	0.987	0.1	0.987	0.2
Peak area %				
Disialo-Tf	0.7	5.9	0.7	6.8
Trisialo-Tf	1.3	9.2	1.4	10.2

Table 4
Repeatability of the CZE determination of disialo- and trisialo-Tf in the ''high CDT'' serum as relative values, calculated on the respective tetrasialo-Tf peak

	Intra-day (n=6)		Day-to-day (n=5)	
	Mean	RSD (%)	Mean	RSD (%)
Migration time				
Disialo-Tf	0.972	0.1	0.971	0.2
Trisialo-Tf	0.987	0.1	0.986	0.1
Peak area %				
Disialo-Tf	6.7	5.9	6.6	6.9
Trisialo-Tf	5.3	8.2	5.5	10.9

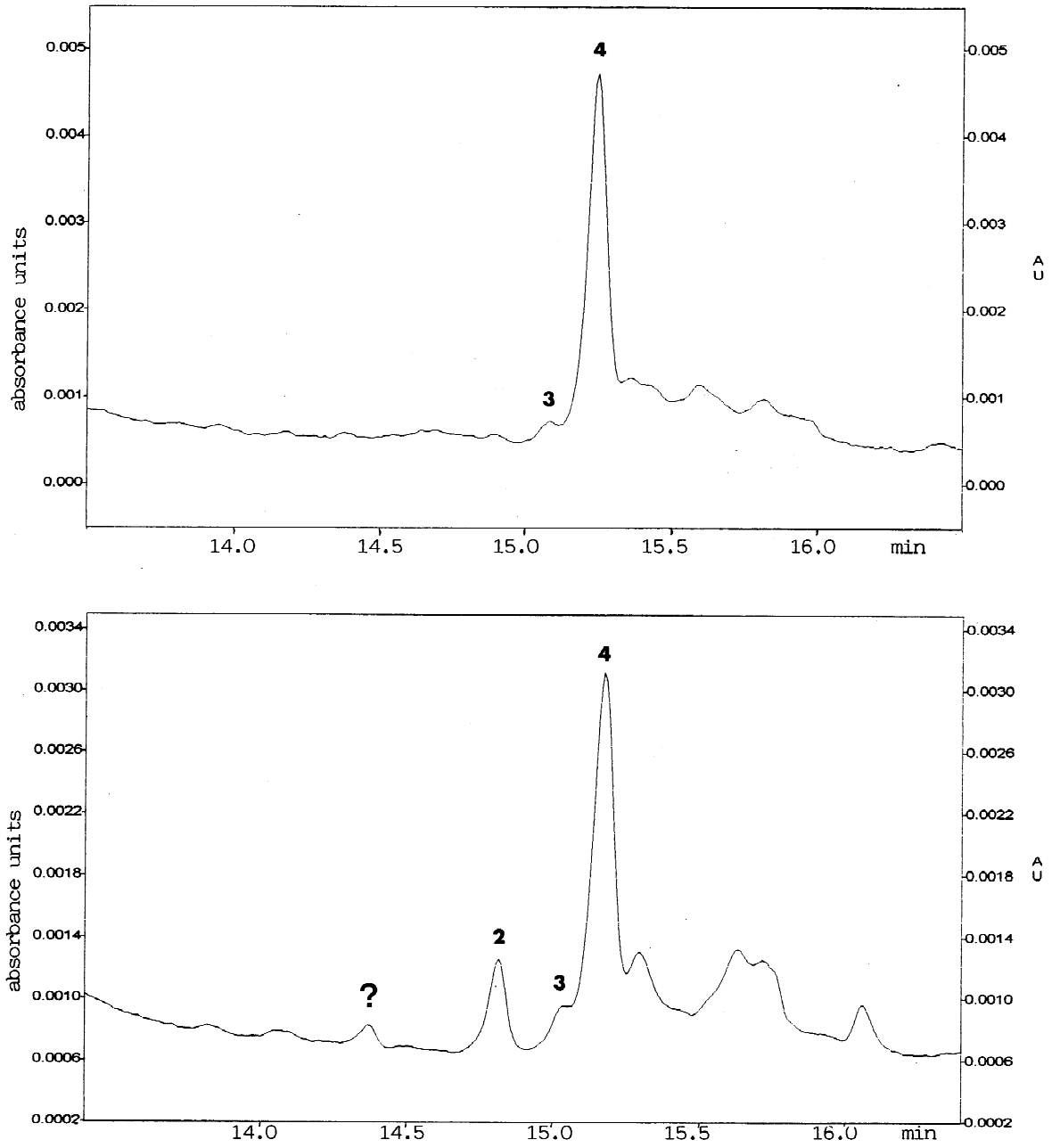

Fig. 5. CZE electropherograms of serum samples from a normal subject (upper) (CDTect: 10 U/l) and an alcohol chronic abuser (CDTect: 97 U/l) (lower). Peaks as in Fig. 1. The question mark indicated a peak with the relative migration time corresponding to asialo-Tf.

centages equal to females, while a clear increase in both genders occurred in alcoholics ($P<0.0001$). Trisialo-Tf was slightly higher in females than in males, but without any statistical significance, in agreement with Martensson et al. [16].

In contrast to these authors who reported no statistically significant changes of trisialo-Tf in alcoholics, our study showed a significant increase of this Tf glycoform ($P<0.01$) in alcohol abusers, although with less significance than disialo-Tf. No

Table 5
CZE analysis of disialo- and trisialo-Tf (as area % of tetrasialo-Tf) performed in sera from selected control subjects and alcoholics (mean±SD)

Subjects		Disialo-Tf	Trisialo-Tf
Controls	Males (35)	1.5±0.7	2.5±1.4
	Females (44)	1.5±0.8	2.6±1.9
	Total (79)	1.5±0.7	2.6±1.7
Alcoholics	Males (21)	6.1±6.0	3.7±2.4
	Females (2)	5.3±1.9	5.7±0.3
	Total (23)	6.1±5.7	3.9±2.4

gender difference could be studied because of the insufficient number of females represented among the alcoholics.

Quite surprisingly, in comparison to the ''normal'' values reported in our previous method [15], the new normal concentrations are significantly higher, particularly for trisialo-Tf. This can be easily explained on the basis of the selection criteria, which in the previous work were based on ''normal'' CDTect results (only male subjects<20 U/l and females<26 U/l were included), while in the present work selection has been based on clinical examination, and haematological and enzymatic values, excluding CDTect levels. Due to an already reported interference of trisialo-Tf with CDT immunoassay [17], the previous criteria could have pre-selected a casework with an erroneously low trisialo-Tf concentration.

A preliminary comparison with CDTect showed 100% confirmation by immunoassay of the samples ($n=10$) with high disialo-Tf (>mean of controls+2 SD) at the CZE assay. Out of eight subjects with high CZE trisialo-Tf (>mean of controls+2 SD), but normal disialo-Tf, six showed increased CDTect concentrations. This is also consistent with the hypothesis of a relevant interference of trisialo-Tf in CDTect immunoassay, as suggested by Arndt et al. [17]. This can be explained with a leakage of trisialo-Tf from the extraction cartridges used in the sample preparation into the assay tubes, leading to increased amounts of Tf like material to be determined by the immunoassay, which employs an antiserum not selective for CDT, but cross reacting with all the Tf isoforms.

This is potentially a major drawback of the immunoassays for CDT determination, because trisialo-Tf, in the recent literature [16] is not considered to be specifically correlated with alcohol abuses.

In the examined casework, it was easily possible to identify D variants of Tf, which also may interfere with immunoassays, giving rise to falsely high CDT values.

4. Concluding remarks

In the present work, the CZE analysis of serum CDT has been substantially improved by addition of a cationic additive to the separation buffer and by increasing the capillary length, thus achieving a better resolution of carbohydrate deficient Tf isoforms from the dominant peak of tetrasialo-Tf. Sensitivity, precision and accuracy have proved to be acceptable for application to real cases.

Because of the relevant social, legal and/or administrative consequences of a diagnosis of alcoholism, which is increasingly based onto CDT analysis [18–20], it is mandatory that biochemical evidences are legally defensible and, for this purpose, the present method seems perfectly adequate to confirm with an independent method CDT results from immunoassays.

CE looks superior to slab gel isoelectric focusing and liquid chromatography, which suffer from several practical drawbacks, including complex operation, poor standardisation, low productivity and high operative costs etc.

CE, indeed, couples the high resolution of electrophoresis in protein separation with the reliability of instrumental analysis and, consequently, could offer an ideal tool for investigating proteins of forensic relevance, such as CDT.

The present work shows also that CZE in highly concentrated borate buffer, in the presence of a cationic additive (DAB), is suitable for the direct determination of the major CDT components in serum, without the need of a complex sample preparation, as it is required by HPLC [9,18] and CZE in polymer network solutions [13,14].

Finally, because of the high versatility of CE, which can be used for a variety of different determinations of clinical and forensic interest, the proposed method seems the most suitable for those laboratories in which CDT assay is not so frequent to

justify the adoption of dedicated instrumentation (like HPLC) or highly trained personnel (like isoelectric focusing).

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