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Dynamically-coated capillaries allow for capillary electrophoretic resolution of transferrin sialoforms via direct analysis of human serum

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Abstract

Transferrin sialoforms with fewer than three sialic acid residues (carbohydrate deficient transferrin; CDT) have been implicated as a marker of certain liver pathologies. Transferrin sialoforms in human sera from alcoholic and non-alcoholic patients was analyzed by capillary electrophoresis (CE) using diaminobutane (DAB) to dynamically-coat the capillary wall to minimize protein–wall interactions. Using a DAB concentration of 3 m*M*, transferrin sialoforms were adequately resolved to allow for direct detection of CDT without extensive treatment of the sera. Serum immunoglobulins, which migrated close to the CDT region, were removed via subtraction with protein A, enhancing the detection of CDT. The reproducibility of sialoform separation in dynamically-coated capillaries was found to be acceptable with run-to-run relative standard deviation values of 0.15% for a sample on a given day and 0.29±0.06% for four samples day-to-day. These results suggest that dynamic-coating approaches may provide a simple alternative to the use of covalently-coated capillaries for the CE separation of complex samples.

Keywords: Dynamic coating; Transferrin; Diaminobutane

1. Introduction

Transferrin (Tf) is a glycoprotein that consists of a 679 amino acid chain with two N-linked carbohydrate chains. As with many glycoproteins, Tf is known to be microheterogeneous as a result of variations in the number of sialic acid residues at the termini of two biantennary carbohydrate chains [1–3]. The total number of sialic acid residues can range from zero to eight and, therefore, as many as nine different sialic acid isoforms or ''sialoforms'' can exist. While the significance of glycosylation-induced microheterogeneity in biology is not entirely understood, subtle changes in the carbohydrate chain of glycoproteins allow for a wide functional diversity. Opdenakker et al. [4] have pointed to microheterogeneity as key in the high fidelity control of numerous cellular functions such as protein transport, folding, and stabilization. While the function of the

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individual sialoforms of Tf is not understood as a whole, the presence of elevated concentrations of Tf with three or less sialic acids (carbohydrate-deficient Tf or CDT) has been shown to correlate with alcoholism and other liver dysfunctions [5–7].

Recent work by our group has focused on the development of capillary electrophoresis (CE)-based assays for CDT as a potential marker for alcoholism and other liver pathologies. Trout et al. [8] demonstrated the direct detection of CDT by resolving the components of serum in a fluorocarbon-coated capillary using a borate buffer containing hydroxyethylcellulose (HEC). Under these conditions, all sialoforms of Tf could be resolved in a 20-min electrophoresis step after direct injection of unpurified serum. While the separation was only qualitative as a result of the complexity of the sample, the resolution and speed of analysis made it a vast improvement over conventional techniques for resolving Tf sialoforms [9–11]. While this CE-based method may set the stage for rapid sialoform profiling of Tf in patient sera, the effectiveness of the method is restricted by the limited longevity of the covalently-coated capillaries used for the separation. Both DB-series [12,13] and fluorocarbon-coated (FC-coated) capillaries [8] have been shown to be effective for Tf sialoform separation, but experience unpredictable loss of functionality with extended use for protein separations. This, combined with the inability to recover functionality once lost, reduce their effectiveness for diagnostic use. In this light, the separation of Tf sialoforms in bare silica capillaries, where surface inactivation is accomplished ''dynamically'' by a surface-active ingredient in the separation buffer, becomes attractive.

Exploitation of dynamic coating procedures for protein CE analysis is not new. In fact, it has been utilized successfully in the separation of many glycoproteins known to be microheterogeneous in nature. Among the numerous additives reported in the literature to passivate the capillary surface [14–22], certain cationic alkanes have been shown to be very effective for dynamically-interacting with the silica surface [17–20]. In particular, dicationic alkanes, such as 1,4-diaminobutane (DAB) and hexamethonium bromide have been shown effective for resolving ovalbumin glycoforms [17,18,21,22] while 1,3-diaminopropane was shown to be effective for resolving the glycoforms of human chorionic gonadotropin [23]. Although the mechanism for such separations is still not completely understood [24,25], it is clear that dual positive charge on the alkyl chain mediates the interaction of the additive with the negatively charged silica surface, thus slowing electroosmotic flow (EOF). Whether the subsequent resolution of glycoprotein components is due solely to this physical phenomenon is still open to question.

In this report, we describe the exploitation of bare silica capillaries dynamically-coated with DAB for the rapid detection of Tf sialoforms following direct injection of serum. This method not only eliminates the need for commercially-coated capillaries, but also provides good run-to-run reproducibility. Since the capillary surface is simply uncoated silica, any fouling was readily reversed using between-run rinses with surface cleansing agents, making regeneration of the capillary surface for dynamic coating simple and fast.

2. Materials and methods

2.1. Materials

Sodium borate and boric acid were obtained from EM Science (Gibbstown, NJ, USA). Ferric citrate was a product of Sigma (St. Louis, MO, USA). Bare silica capillaries were obtained from Microtechnologies (Phoenix, AZ, USA). Diaminobutane was purchased from Acros (Geel, Belgium). Affi-Gel protein A agarose was purchased from Bio-Rad (Hercules, CA, USA).

2.2. Buffer preparation

All buffers were prepared with Milli-Q (Millipore, Bedford, MA, USA) ultra-purified water. Borate buffer (100 m*M*) was prepared by titrating 25 m*M* sodium borate with 100 m*M* boric acid until pH 8.5 was obtained. Borate buffer with DAB was prepared from a stock solution of 26 m*M* DAB in borate buffer. The stock solution and borate buffer were stored at 4°C. Before use, the DAB buffer was brought to room temperature and diluted with borate buffer to a 3 m*M* concentration of DAB.

2.3. Capillary electrophoresis

CE separation was carried out using a Hewlett-Packard 3D-CE system equipped with a UV diode array detector. A HP Kayak computer utilizing Chemstation software was used for instrument control and data collection. All peak information was obtained using the Chemstation software. New capillaries were conditioned with high-pressure rinses (0.9 bar) of 1 *M* NaOH followed by water until a stable baseline was obtained. For a typical analysis the following method was used: a 3 min rinse with 0.5 *M* NaOH, a 6 min rinse with 500 m*M* borate buffer, a 5 min rinse with the 3 m*M* DAB solution in borate buffer, sample injection at 50 mbar for 10 s, followed by a 5 s injection of separation buffer. Separation was carried out at 25 kV (constant voltage) for 25 min, using normal polarity (inlet was anode), a typical current of approximately 15 μA was observed in all experiments. Capillaries, 64.5 cm (56 cm to detector)×50 μm I.D., were maintained at 20°C. Detection was measured by absorbance at 200 nm. Peak identity was determined by spiking experiments with transferrin standards.

2.4. γ-Globulin subtraction

Removal of serum immunoglobulin G (IgG) was carried out to determine if co-elution of IgG interfered with CDT detection. The agarose gel protein A slurry (100 μl) was washed by diluting with 100 μl of phosphate-buffered saline, then aspirating a number of times using a micropipette. The dilute slurry was spun down and 100 μl of supernatant was removed. To the remaining material, 100 μl of 500 μ*M* ferric citrate was added. This mixture was again aspirated and spun down followed by the removal of 100 μl of supernatant. Human serum (40 μl) was added to the protein A slurry and was mixed for 5 min with a vortex-mixer. After mixing, 660 μl of ferric citrate was added and the solution spun down for a third time. An aliquot (80 μl) of the supernatant was used for analysis.

2.5. Controls

Positive controls consisted of iron-saturated holotransferrin for normal separations, and human IgG for subtraction with protein A. Normal serum was used as a negative control.

3. Results and discussion

3.1. Optimizing conditions for Tf separation in uncoated capillaries

Oda et al. [21] demonstrated that the addition of 1 m*M* DAB to borate buffer allowed for the efficient electrophoretic separation of ovalbumin glycoforms in 45 min. To test whether similar conditions would allow for Tf sialoform resolution, purified holotransferrin was electrophoresed in a borate buffer augmented with DAB ranging in concentration from 0 to 4 m*M* (Fig. 1). In the presence of 1 m*M* DAB, the sialoforms begin to resolve, although poorly in comparison with buffer devoid of the additive. Improved resolution is observed at concentrations higher than 1 m*M*, with optimal separation at 3–4 m*M*. At concentrations higher than 4 m*M*, band broadening begins to become an issue. It is clear from these results that there is a trade-off at higher DAB concentrations between improved resolution and the extended analysis times that are observed with lower signal-to-noise (S/N) ratios. Table 1 summarizes the effect of DAB concentrations on these three parameters. A 10-fold increase in DAB concentration improves the resolution of the tetra- and pentasialo Tf, but with a three-fold increase in migration time and a six-fold decrease in detection sensitivity (S/N ratio). Since resolving the sialoforms is of primary concern, the best combination of sialoform resolution, detection sensitivity and analysis time was deemed to be with DAB at a concentration of 3 m*M*. This was used for subsequent experiments.

3.1.1. Dynamically- versus covalently-coated capillaries for sialoform separation

Covalently-modified capillaries have been shown to be adequate for resolving Tf sialoforms under the appropriate buffer conditions [8]. However, under these conditions, these capillaries experience a degradation in performance over time and have a limited lifetime. Consequently, they are not likely to provide robust performance for high throughput analysis with

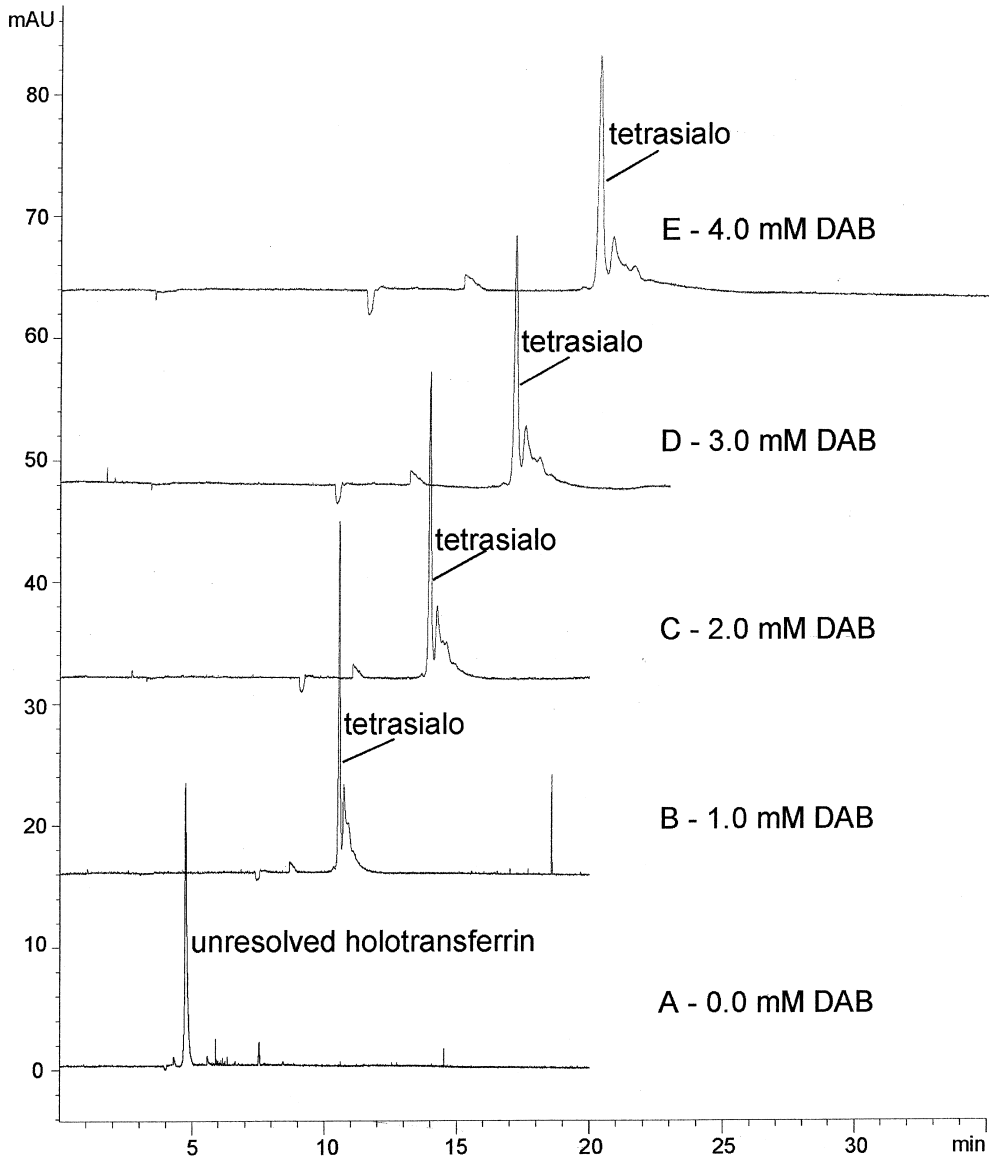

Fig. 1. Capillary electrophoresis of holotransferrin with various concentrations of buffer additive. Holotransferrin (1 mg/ml) diluted 1:20 in 500 μ*M* ferric citrate. Separations were carried out in a 64.5 cm×50 μm bare silica capillary containing 100 m*M* borate buffer, pH 8.5 with 0.0 to 4.0 m*M* DAB at 25 kV with the capillary thermostated at 20°C. All samples were pressure-injected at 50 mbar for 10 s. Detection was at 200 nm. Separation was performed using a Hewlett-Packard 3D-CE system controlled by a HP Kayak computer utilizing Chemstation software.

protein based separations. Fig. 2 provides a direct comparison of the electrophoretic analysis of normal serum in an FC-coated capillary (Fig. 2A) and that in a capillary dynamically-coated with DAB (Fig. 2B). Peak numbers in this and subsequent figures indicate the number of sialic acid residue termini on the glycoprotein. With an FC-coated capillary and a borate-based buffer containing a polymer network, a reversed polarity separation (inlet – anode, outlet – cathode) is required providing albumin with the

Table 1
Comparison of signal-to-noise ratio and resolution as a function of DAB concentration

[DAB] (m*M*)	Migration time (tetrasialo Tf) (min)	Resolution[a] (tetrasialo Tf/pentasialo Tf)	Signal-to-noise ratio (tetrasialo Tf)
0.5	8.460	0.83	164.5
1.0	10.563	0.84	113.2
1.5	12.464	0.91	78.8
2.0	13.993	0.92	70.8
3.0	17.201	0.95	42.5
4.0	20.374	1.00	40.9
5.0	23.319	1.05	28.7

[a] Resolution calculated by Chemstation software on Hewlett-Packard Kayak computer.

fastest migration, followed by the α_1, α_2, β and γ region proteins. Due to the extensive time required for the γ-globulins to reach the detector, the separation was terminated at 20 min. With DAB-coated capillaries, separations are carried out under normal polarity conditions, yielding migration order that is reversed in comparison with the FC-capillary separation, with the γ-globulins (γ region) migrating fastest followed by the Tf sialoforms (β region) (Fig. 2A). The use of dynamically-coated capillaries presents a slight advantage over the method of Trout et al. [8]. In addition to the elimination of the need for covalently-coated capillaries, the proteins of interest (Tf sialoforms) reach the detector before the albumin, α_1 and α_2 proteins. This is offset, however, by the proximity of the CDT region to the γ-globulins which complicates the profile. In addition, one must be concerned by the fact that a complex protein mixtures is being directly injected into bare silica capillary at neutral pH – conditions that are ideal for fouling of the capillary surface. Reproducibility was evaluated by executing consecutive injections of serum and calculating the relative standard deviation (RSD) values for the migration time (t_m) of the tetrasialo Tf peak. With eight consecutive injections of serum diluted 1:20 in 500 μ*M* ferric citrate, the tetrasialo Tf had a mean t_m of 20.16±0.38 min associated with a RSD value of 0.15%. When serum was electrophoresed on different days, the RSD value (for $n=4$) was acceptable at 0.30±0.06%. It is noteworthy that the interaction of DAB with the capillary wall is rapid and reversible. Rinsing the capillary with a buffer containing DAB and then electrophoresing in the same buffer devoid of DAB yielded a separation equivalent to that in Fig. 1A. This indicates that dynamic deactivation of the silica surface is solely a function of the DAB concentration in the buffer, as well as the ionic strength of the buffer. This is advantageous since rapid regeneration of the capillary surface can be achieved.

3.2. Direct CE analysis of non-alcoholic and alcoholic sera

Using optimal CE conditions (defined as 3 m*M* DAB, 100 m*M* borate buffer, pH 8.5, 25 kV), the components in non-alcoholic and alcoholic sera were electrophoresed to determine whether obvious differences in the β-region proteins of serum could be detected, specifically an elevation in the relative amount of the diasialoform. To ascertain that iron saturation of the Tf sialoforms was complete, all samples were prepared by diluting the serum 1:20 in 500 μ*M* ferric citrate [8]. Transferrin binds two Fe^{3+} ions, which need to be saturated in order to reduce the complexity of the transferrin profile. Failure to saturate the iron-binding sites is identified by doublet peaks in the transferrin profile. A further discussion of the iron saturation can be found in Ref. [8]. Fig. 3 shows the electropherograms resulting from electrophoresis of sera from three normal (non-alcoholic) individuals, where the tetrasialoform is present at the highest concentration with the penta-, hexa-, tri- and disialoforms at lower levels. The relative amount of disialoform, determined by peak area normalization (peak area/migration time), in comparison with the total normalized peak area for di-, tri-, and tetrasialoforms combined was 2.01±0.82% ($n=9$). While Trout et al. [8] demonstrated that the lower

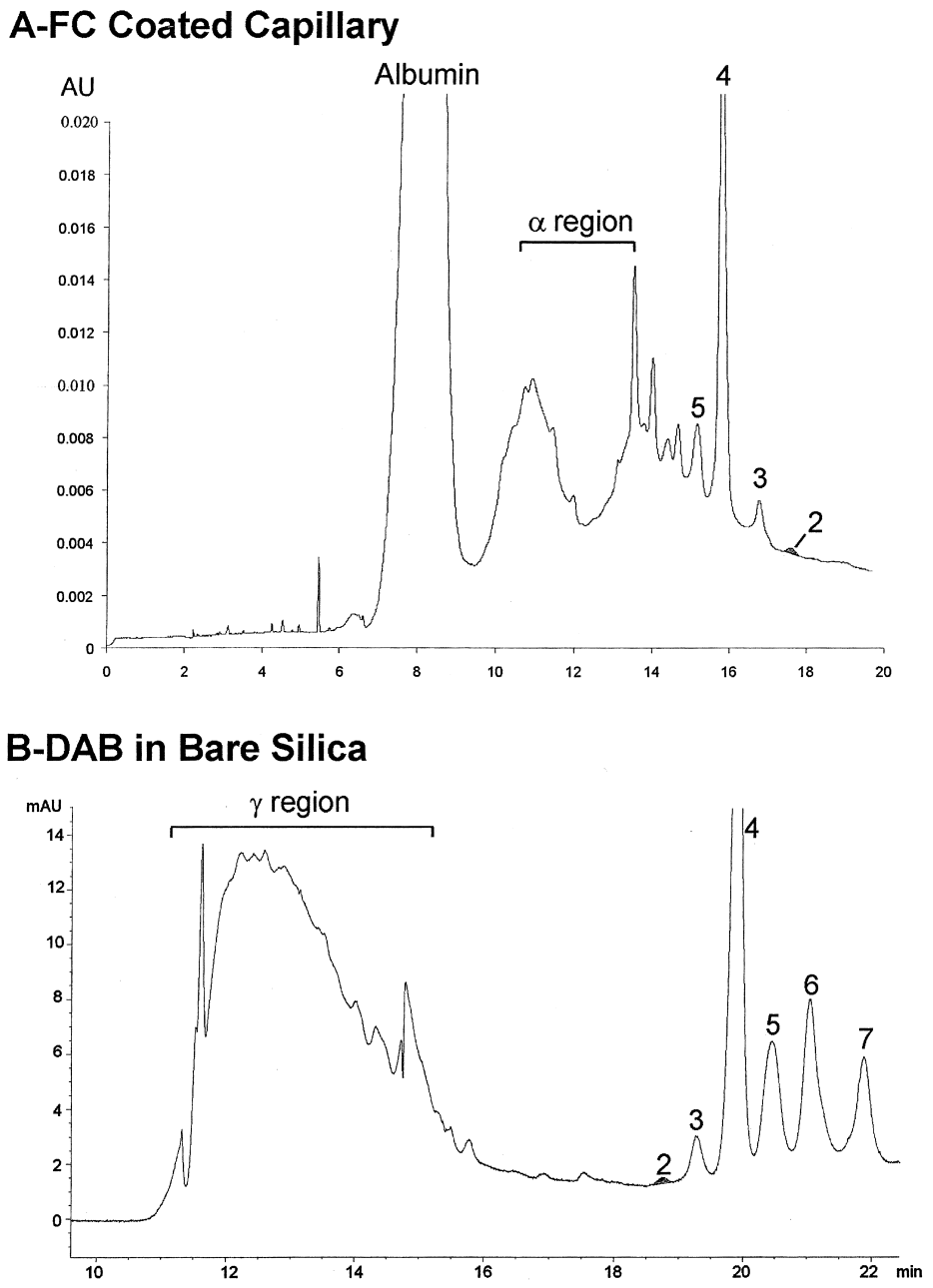

Fig. 2. Capillary electrophoresis of non-alcoholic patient sera in FC capillary and bare silica capillary. (A) Non-alcoholic patient serum. Peaks are labeled with numbers corresponding to the terminal sialic acid residues on the carbohydrates (peak 2 is disialoform, peak 3 is trisialoform, peak 4 is tetrasialoform). Separation was carried out in a 37 cm×50 μm FC capillary containing 100 m*M* borate buffer, pH 8.5, with 0.5% HEC at 15 kV with the capillary thermostated to 20°C. Sample was pressure-injected at 35 mbar for 40 s. The γ region is not seen on this time scale. Separation was performed using a Beckman Coulter P/ACE System 5510 controlled by an IBM (486) computer utilizing System Gold software (Version 8.1). (B) Non-alcoholic patient serum. Separation was carried out in a 64.5 cm×50 μm bare silica capillary containing 100 m*M* borate buffer, 3.0 m*M* DAB, pH 8.5 at 25 kV with the capillary thermostated at 20°C. Sample was pressure injected at 50 mbar for 10 s. The α region and albumin are not seen on this time scale. Separation was performed using a Hewlett-Packard 3D-CE system controlled by a HP Kayak computer utilizing Chemstation software. Peak 2 is marked in black for ease of viewing.

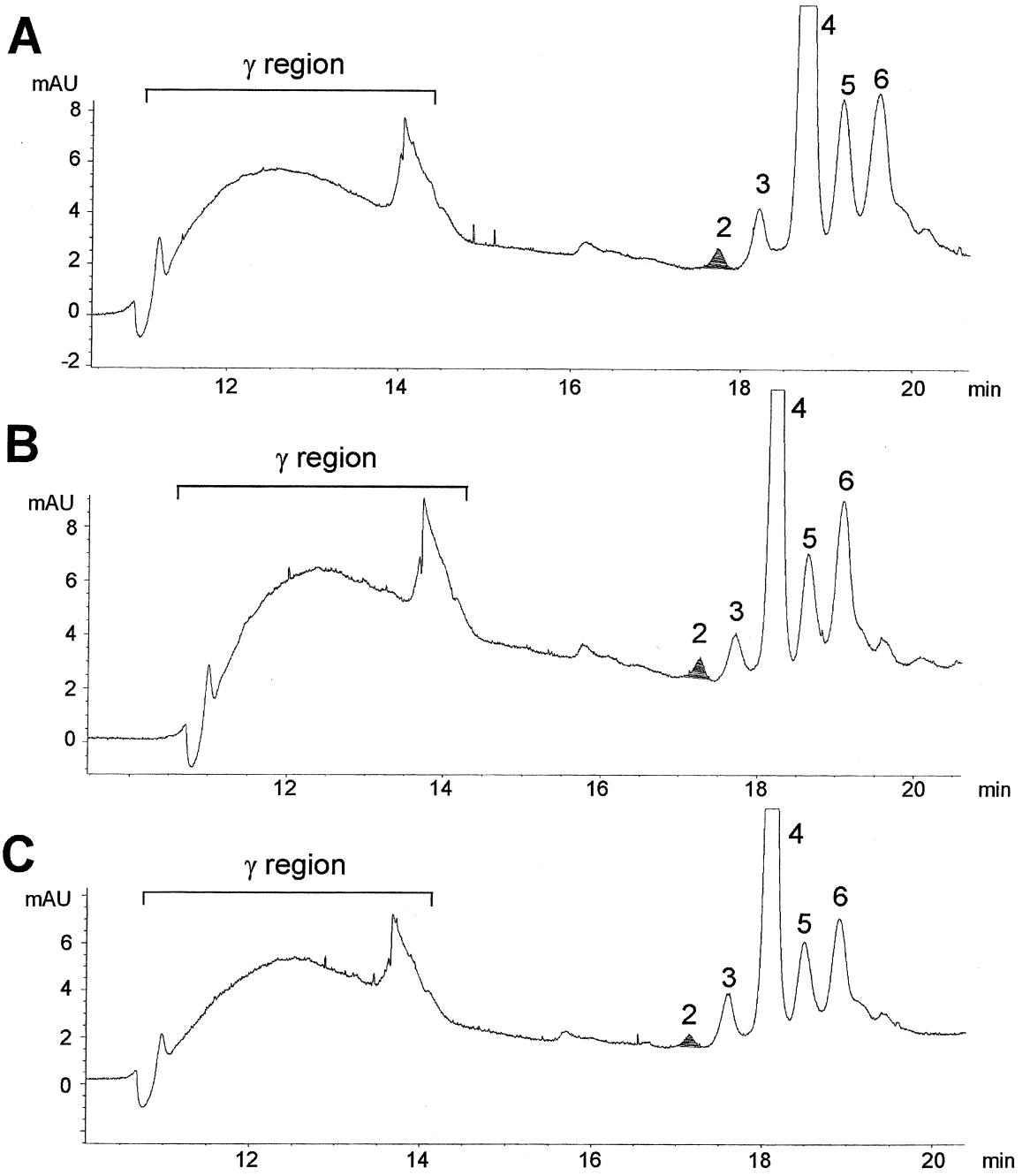

Fig. 3. Capillary electrophoresis of non-alcoholic patient sera. Non-alcoholic sera of three patients. Peaks are labeled with numbers corresponding to the terminal sialic acid residues on the carbohydrates (peak 2 is disialoform, peak 3 is trisialoform, peak 4 is tetrasialoform). Separations were carried out in a 64.5 cm×50 μm bare silica capillary containing 100 m*M* borate buffer, 3.0 m*M* DAB, pH 8.5 at 25 kV with the capillary thermostated at 20°C. Sample was pressure-injected at 50 mbar for 10 s. Peak 2 is marked in black for ease of viewing. Separation was performed using a Hewlett-Packard 3D-CE system controlled by a HP Kayak computer utilizing Chemstation software.

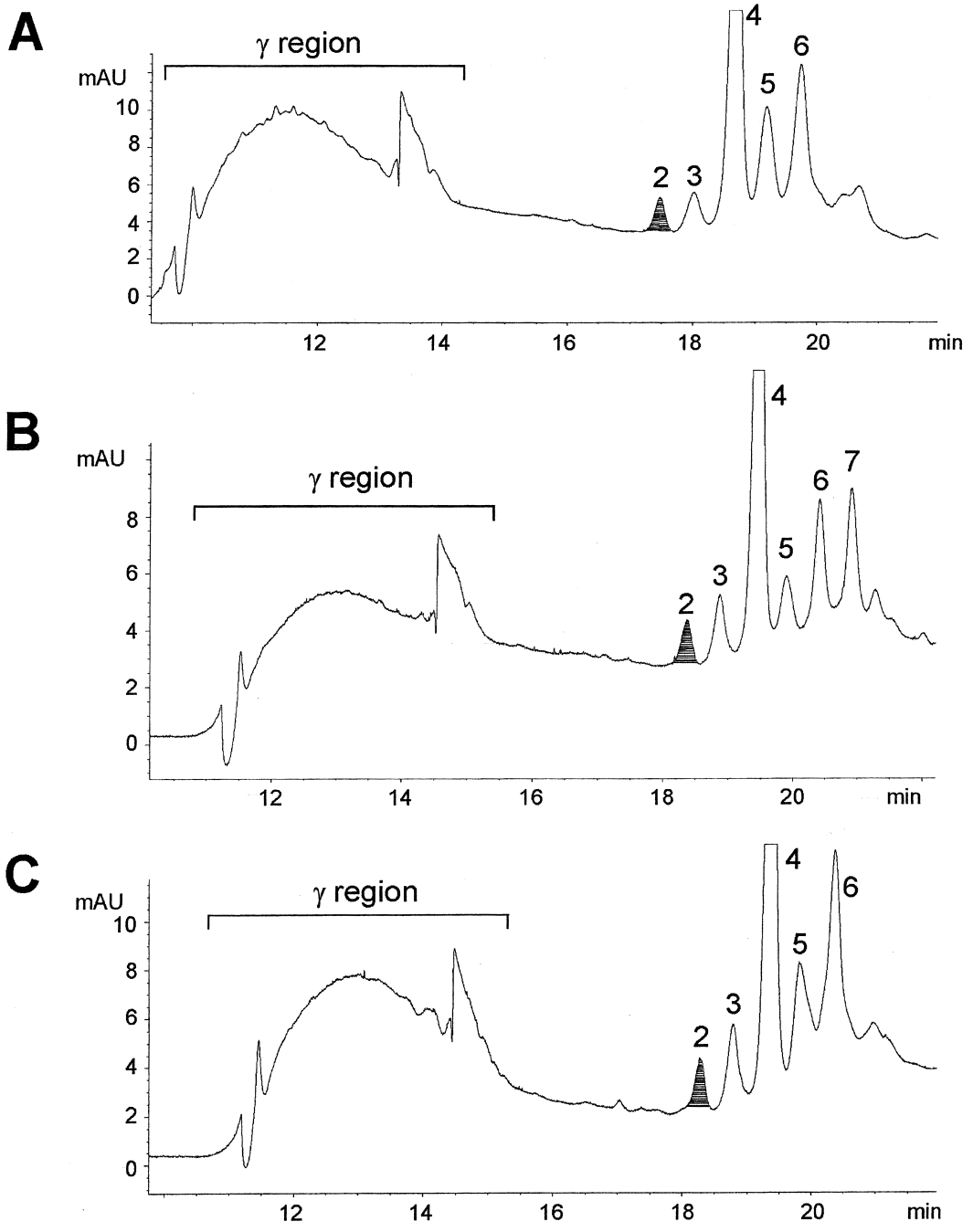

Fig. 4. Capillary electrophoresis of alcoholic patient sera. Alcoholic sera of three patients. Peaks are labeled with numbers corresponding to the terminal sialic acid residues on the carbohydrates (peak 2 is disialoform, peak 3 is trisialoform, peak 4 is tetrasialoform). Separations were carried out in a 64.5 cm×50 μm bare silica capillary containing 100 m*M* borate buffer, 3.0 m*M* DAB, pH 8.5 at 25 kV with the capillary thermostated at 20°C. Sample was pressure injected at 50 mbar for 10 s. Peak 2 is marked in black for ease of viewing. Separation was performed using a Hewlett-Packard 3D-CE system controlled by a HP Kayak computer utilizing Chemstation software.

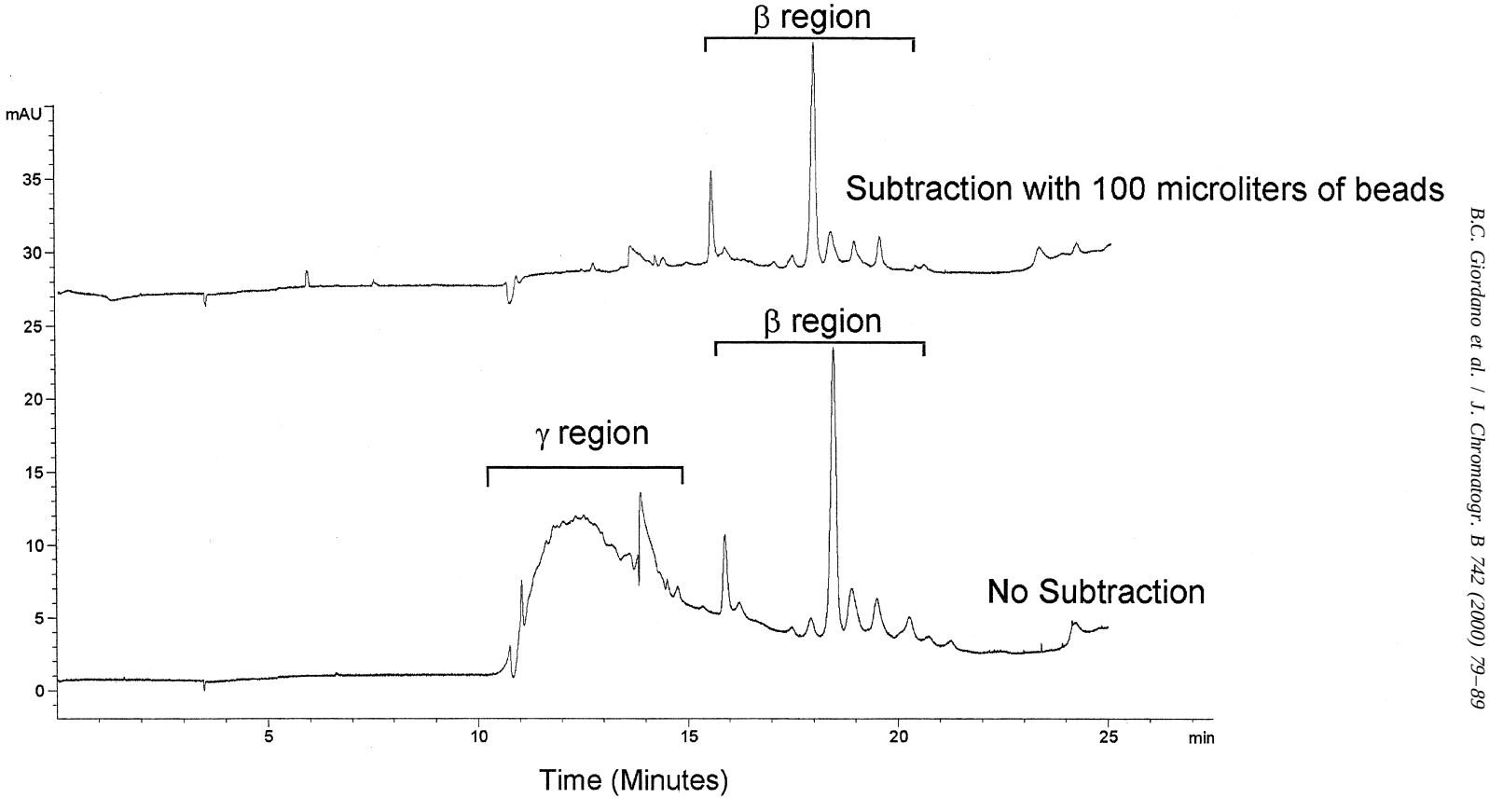

Fig. 5. Determination of γ-globulin subtraction effectiveness. Liver dysfunction patient serum. Subtraction of the γ-globulin region was performed as described in Section 2.4. Separation was carried out in a 64.5 cm×50 μm bare silica capillary containing 100 m*M* borate buffer, 3.0 m*M* DAB, pH 8.5 at 25 kV with the capillary thermostated at 20°C. Sample was pressure injected at 50 mbar for 10 s. Separation was performed using a Hewlett-Packard 3D-CE system controlled by a HP Kayak computer utilizing Chemstation software.

sialoforms could be immunosubtracted from the profile using anti-Tf antibodies, it cannot be ascertained that other proteins are not present in the region where the higher sialoforms (hexa- and heptasialoforms) migrate. Hence, the observed fluctuations in this region may not directly reflect elevation in the penta- and hexasialoform concentration.

Stibler et al. [26] have suggested a link between elevated CDT (the presence of the lower sialoforms including the disialoform) and alcohol abuse. They found that elevation of the disialoform corresponded to intake in excess of 60 g/day of ethanol [27]. Fig. 4 illustrates that elevated levels of the disialo Tf in alcoholic sera (serum from patients prescreened for alcohol abuse) can be observed under these conditions. The relative amount of the disialoform (to the total amount of di-, tri- and tetra-) was calculated to be 6.98±2.42% for a sample data set (n=6), representing a significant difference from the levels in non-alcoholic sera. Of course, the current method offers only a semi-quantitative evaluation of Tf sialoforms in serum, but the ability to separate Tf sialoforms in a bare silica capillary in less than 25 min, without extensive pretreatment of the serum, could offer a significant improvement on existing techniques for the CDT analysis. This is bolstered by the advantage that, in contrast to slab gel-based techniques, detection is on-line via absorbance. Consequently, problems with dyes that bind aberrantly to proteins that are glycosylated are avoided, conveying a distinct advantage to the CE-based methodology.

3.3. γ-Globulin subtraction

As alluded to earlier, one potential problem with this assay was the proximity of the migrating Tf sialoforms to the γ-globulin region. While this may not be problematic with detection of the disialo Tf, the ability to detect mono- or asialo Tf would definitely be compromised. We sought to remove the γ-globulins from the serum using a procedure similar to that used for identifying and typing monoclonal proteins in serum [28]. Protein A, covalently bound to agarose gel beads, is known to effectively bind to the F_c region of γ-globulins [29] and, therefore, may be effective in clarifying the CDT region. A number of concentrations were tested by varying the volume of protein A slurry used in the subtraction (data not shown). As described in Section 2.4, γ-globulin subtractions were performed using volumes of slurry ranging from 25 μl to 200 μl. The use of a slurry volume in excess of 100 μl was not found to improve the subtraction process. Fig. 5 illustrates the effectiveness of protein A subtraction of the γ-globulins present in the serum from a patient with compromised liver function (as determined by liver enzyme tests). The CDT region is effectively clarified of γ-globulins which potentially could interfere. The percent disialoform relative to the total of the tetra-, tri-, and disialoforms, was 1.48% for a single run prior to protein A subtraction. The average amount post-subtraction was 1.81±0.10%, for subtractions performed on the same sample (n=3). While the absolute amount of transferrin decreased slightly, the relative percentages remained comparable. The subtraction of γ-globulins may prove to be useful in more complex sera such as that observed in patients with extremely compromised liver function [8].

4. Concluding remarks

The results of this study illustrate that the use of dynamic coating of a capillary with DAB is effective for resolving human transferrin sialoforms. The dynamic inactivation of the capillary surface is apparently effective for minimizing or even eliminating protein–wall interaction as evidenced by the ability to directly electrophorese serum with good reproducibility. This not only provides another tool for protein analysis by CE, but may allow for simple and reproducible transferrin profiling to be done with little or no sample preparation. Consequently, the ability of CE for direct on-line detection can be exploited to circumvent the problems associated with gel-based detection using protein dyes. While the quantitative aspects of this method must be developed for accurate quantification of CDT in alcoholic sera, the results presented here demonstrating visible differences between normal and alcoholic sera, illustrate the potential of this method for rapid and simple CE-based sialoform profiling.

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