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Effect of buffer constituents on the determination of therapeutic proteins by capillary electrophoresis

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ABSTRACT

Capillary electrophoresis has proved to be a versatile method for the determination of proteins, peptides and amino acids in pharmaceutical formulations. For quantification of the capillary electrophoresis data, however, significant errors may result if the analysis is performed using improper separation conditions. The peak area response for protein analytes, which is generally low in conventional UV detection, may also vary dramatically depending on the nature of the buffer used in the separation. This paper describes the effects of various buffer constituents and analytical conditions on the capillary electrophoretic separation and quantification of a humanized monoclonal antibody in bulk form and in a typical therapeutic formulation. For optimum peak area response and reproducibility, protein derivatization with an appropriate chromophore (*e.g.*, fluorescamine) and separation in the presence of a moderate ionic strength buffer containing lithium chloride, tetramethylammonium chloride or trimethylammonium propylsulfonate is recommended. General guidelines for the determination of proteins by capillary electrophoresis and a rationale for the use of internal standards to improve the quantification of data are also discussed.

INTRODUCTION

Capillary electrophoresis (CE) is an analytical technique capable of yielding remarkable information in a variety of applications, especially in the analysis of proteins and peptides [1–5]. Nevertheless, the technique, in general, has not yet achieved the same degree of acceptance as more conventional procedures, such as high-performance liquid chromatography and conventional gel electrophoresis. This is partly due to the fact that despite the enormous resolving power of CE, quantification of data has encountered numerous problems (especially in the separation of protein macromolecules). These include the potential adsorption of the protein analyte to the capillary walls (which often gives rise to band broadening and low recovery of the separated protein analyte), and the necessity to optimize the running conditions to maximize the yield and degree of separation.

Numerous efforts have been made to separate proteins by free-solution CE. Some of these separation optimization methods include coating of the capillary surface, changing the pH of the separation buffer and the addition of additives to the separation buffer [6–23]. However, most efforts have been directed to improving the separation profile and very little attention has been placed on the quantitative aspects of the separation.

In a previous paper [24], we demonstrated the importance of temperature in the separation and determination of protein drug substances present in a solution mixture. As little as a 5°C variation in temperature was found to have a critical effect on the separation profile and stability of the drug substance (recombinant interleukin-1α). In turn, the quantitative profile was also affected by chemical changes produced in the protein (*i.e.*, deamidation).

In this work, we have tried to develop a better understanding of how chemical factors, such as the presence of certain salts or zwitterions in the run-

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ning buffer, influence the performance of CE in the determination of a protein drug substance. We have selected the humanized monoclonal antibody[a] anti-TAC [25] as a model protein and have developed the conditions for CE analysis by approaches similar to those previously described for proteins not specifically targeted for therapeutic use [12–14]. In addition, internal standards were used in the CE separation of the monoclonal antibody to monitor changes affecting the quantitative profile. In order to enhance the detection sensitivity, and also to improve analyte resolution, samples were derivatized with the chromophore fluorescamine prior to CE analysis. The proposed reaction scheme of fluorescamine with primary and secondary amines has been discussed previously [26,27] and is shown in Fig. 1.

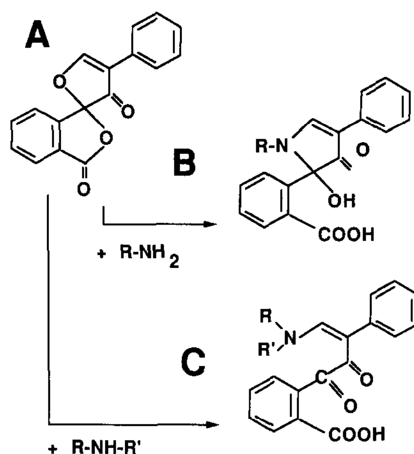


Fig. 1. Schematic representation of the molecular structure of (A) fluorescamine, (B) the derivatized reaction product involving a reacting primary amine functional group-containing analyte and (C) the derivatized reaction product involving a reacting secondary amine functional group-containing analyte.

EXPERIMENTAL

Reagents and samples

All chemicals were obtained at the highest purity available from the manufacturer, and were used without additional purification. Sodium hydroxide, sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$), lithium chloride, N-acetyltryptophan and fluorescamine were purchased from Sigma (St. Louis, MO, USA), tetramethylammonium chloride and L-arginine from Fluka (Ronkonkoma, NY, USA), trimethylammonium propylsulfonate from Waters–Millipore (Milford, MA, USA), acetone (HPLC grade), pyridine (Fisher Certified) and hydrochloric acid solution (12 *M*) from Fisher Scientific (Fair Lawn, NJ, USA), and purified bulk drug substance (humanized anti-TAC monoclonal antibody) from Hoffmann-La Roche (Nutley, NJ, USA). Reagent solutions and buffers were prepared using triply distilled, deionized water, and routinely degassed and sonicated under vacuum after filtration.

Millex disposable filter units (0.22 μm) were purchased from Millipore (Bedford, MA, USA) and fused-silica capillary columns from Scientific Glass Engineering (Austin, TX, USA) and Polymicro Technologies (Phoenix, AZ, USA).

Instrumentation

A commercially available CE instrument (P/ACE System 2000, Beckman Instruments, Palo Alto, CA, USA) was used. The instrument, containing Beckman system software, was controlled by an IBM PS/2 Model 50-Z computer. Samples were stored in a microapplication vessel assembly, consisting of a 150-μl conical microvial inserted into a standard 4-ml glass reservoir and held in position for injection by an adjustable spring. In order to minimize evaporation of the sample volume (100 μl), about 1–2 ml of cool water was added to the microapplication vessel housing the microvial. The external water serves as a cooling bath for the sample in the microvial and as a source of humidity to prevent sample evaporation and concentration. After insertion of the microvial, the microapplication vessel assembly was covered with a rubber injection septum and placed in the sample compartment of the CE instrument. Samples were injected by pressure and the data acquisition and analysis were carried out using System Gold chromatography software (Beckman Instruments, San Ramon, CA, USA). Data integration was also carried out with a Model D-2500

[a] Humanized monoclonal antibody anti-TAC is an IgG-class genetically engineered hybrid antibody, containing approximately 90% human sequence and 10% murine sequence. The antibody is directed against, and is specifically recognized by, the human receptor for interleukin-2, which is a well characterized lymphokine involved in the complex network of cellular communications [25].

Chromato-integrator (Hitachi Instruments, Danbury, CT, USA)

Sample preparation

Stock solutions were individually prepared by dissolving L-arginine (50 mg/ml) and N-acetyltryptophan (1 mg/ml) in 0.1 *M* sodium tetraborate buffer (pH 9.0). Purified bulk drug anti-TAC (6.7 mg/ml) was prepared in phosphate buffer (pH 7.0) and a typical liquid formulation dosage of anti-TAC (6.0 mg/ml) was prepared in the same buffer. Concentrated solutions were diluted to their specified working strengths with the same sodium tetraborate buffer.

Sample derivatization

For CE analysis without fluorescamine derivatization, assay samples were diluted to the desired concentrations with sample dilution buffer [0.1 *M* sodium tetraborate buffer (pH 9.0)] to a total reaction mixture of 100 μl, and directly transferred into the conical vial and then inserted in the microapplication vessel assembly on the CE instrument.

For CE analysis of fluorescamine derivatives, solutions of the respective analyte samples (*i.e.*, humanized anti-TAC monoclonal antibody concentration ranging from 33.5 to 335 μg or from 0.22 to 2.23 pmol per 100 μl of reaction mixture) were transferred into a 500-μl microcentrifuge tube and their total volume was adjusted to 70 μl by addition of sample dilution buffer. Derivatization was performed by the addition of 30 μl of fluorescamine solution (3 mg/ml of fluorescamine in acetone containing 20 μl of pyridine) to the sample while continuously and vigorously vortex mixing. After *ca.* 2 min, the contents of the microcentrifuge were transferred into the conical microvial and then inserted in the microapplication vessel assembly for analysis.

Operating conditions

Sample solutions for analysis in microapplication vessels were placed in the sample holder of the analyzer. The analysis program was initiated and the first sample automatically injected into the capillary by a positive nitrogen pressure of 0.5 p.s.i. (3500 Pa) for 5 s. At the completion of each run, the capillary column was sequentially washed by injection of 2.0 *M* sodium hydroxide solution, 0.1 *M* sodium hydroxide solution and distilled, deionized water, and then regenerated with running buffer.

The CE separations reported were performed using four different buffers: (1) 0.05 *M* sodium tetraborate buffer (pH 8.3) containing 0.025 *M* lithium chloride; (2) 0.05 *M* sodium tetraborate buffer (pH 8.3) containing 0.025 *M* tetramethylammonium chloride; (3) 0.05 *M* sodium tetraborate buffer (pH 8.3) containing 0.025 *M* trimethylammonium propylsulfonate; and (4) 0.05 *M* sodium tetraborate buffer (pH 8.3) containing 0.50 *M* trimethylammonium propylsulfonate. The CE instrument was equipped with a 70 cm (63 cm to the detector) × 75 μm I.D. capillary column. The CE separation was performed at 29 kV. The capillary temperature for all experiments was maintained at 25°C during the run. Under these conditions, *ca.* 30 nl (6 nl/s) were injected into the capillary column [28]. Monitoring of the analytes was performed at 214 nm.

RESULTS

Fig. 2 depicts the electropherograms of underivatized and derivatized humanized anti-TAC monoclonal antibody. The therapeutic antibody was analyzed in the presence of the commonly used parenteral excipients L-arginine and N-acetyltryptophan. As shown in Fig. 2A, the underivatized analytes were well separated from each other. L-Arginine (peak 1) migrated very fast, followed by anti-TAC (peak 2) and N-acetyltryptophan (peak 3). The fluorescamine-derivatized analytes (Fig. 2B), L-arginine (peak 2), N-acetyltryptophan (peak 4) and anti-TAC (peak 5), were also separated well from each other and from the peaks corresponding to the constituents of the derivatization reagent, fluorescamine (peak 6) and the organic solvents acetone and pyridine (comigrating at peak 1). Derivatization with fluorescamine was observed to have a marked effect on analyte mobility and peak area in CE analysis. As shown in Fig. 2B and Table I, derivatized L-arginine and anti-TAC migrated slower than their underivatized counterparts. For N-acetyltryptophan, however, the mobility and peak area were unchanged after fluorescamine derivatization. This suggests that the potentially reactive amine group was blocked, possibly by steric hindrance, and was unable to react with the reagent.

The linearity of the anti-TAC peak area as a

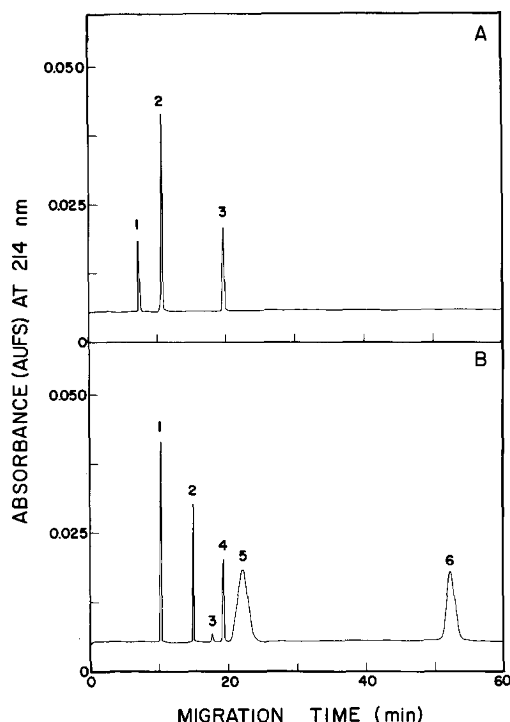


Fig. 2. Capillary electrophoresis profile of underivatized and fluorescamine-derivatized analytes. (A) Electropherogram of underivatized L-arginine (peak 1), anti-TAC (peak 2) and N-acetyltryptophan (peak 3). (B) Electropherogram of fluorescamine-derivatized analytes. Peaks: 1, acetone; 2, L-arginine; 3, unknown; 4, N-acetyltryptophan; 5, anti-TAC; 6, fluorescamine reagent. The separation buffer was 0.05 *M* sodium tetraborate buffer (pH 8.3) containing 0.025 *M* LiCl. The concentrations of analytes, per 100 μl of reaction mixture, used in this experiment were as follows: (1) underivatized analytes, L-arginine 1000 μg (5.74 nmol), bulk anti-TAC monoclonal antibody 201 μg (1.34 pmol) and N-acetyltryptophan 10 μg (40.6 pmol); (2) derivatized analytes, L-arginine 10 μg (57.0 pmol), bulk anti-TAC monoclonal antibody 201 μg (1.34 pmol) and N-acetyltryptophan 10 μg (40.6 pmol).

function of concentration was investigated with and without fluorescamine derivatization. The reaction was carried out in the presence of fixed concentrations of internal standards and increasing concentrations of anti-TAC. As shown in Fig. 3, the peak area for the derivatized anti-TAC monoclonal antibody increased linearly with increasing concentration over the range 0.22–2.23 pmol per 100 μl of reaction mixture. The response curve was slightly sigmoidal in shape. For underivatized anti-TAC, the peak area also increased linearly with increasing concentration up to about 1.79 pmol per 100 μl of reaction mixture, but then reached a response plateau above which no further increase was observed. The plateau in the peak area suggests that the solubility of the underivatized anti-TAC is decreased in the running buffer, possibly owing to the presence of LiCl, and at concentrations above 1.79 pmol per 100 μl of reaction mixture (2.68 mg/ml) the analyte may be precipitating from solution.

The effects of the running buffer additives tetramethylammonium chloride (TMAC), trimethylammonium propylsulfonate (TMAPS) and lithium chloride (LiCl) on the separation and quantification of the anti-TAC monoclonal antibody were investigated. The molecular structures of TMAC and TMAPS are shown in Fig. 4. TMAC, a quaternary salt, has been demonstrated to be an effective agent in preventing adsorption of macromolecules to glass [29,30]. Similarly, TMAPS, a zwitterion, has been used to prevent the binding of proteins to fused-silica capillary columns [31]. Neutral salts, *e.g.*, LiCl, have also been found to stabilize tertiary structures of some proteins in solution [30,32]. As shown in Table II, TMAC, TMAPS and LiCl were

TABLE I

EFFECT OF FLUORESCAMINE ON THE MIGRATION TIME AND PEAK AREA OF HUMANIZED ANTI-TAC MONOCLONAL ANTIBODY AND INTERNAL STANDARDS

For this experiment, the data were obtained by using the following concentrations of the various analytes (in 100 μl of reaction mixture): (1) native (underivatized), L-arginine 20 μl (50 mg/ml); bulk anti-TAC 30 μl (6.7 mg/ml), N-acetyltryptophan 10 μl (1 mg/ml); (2) fluorescamine-derivatized analytes, L-arginine 10 μl (1 mg/ml), bulk anti-TAC 30 μl (6.7 mg/ml), N-acetyltryptophan 10 μl (1mg/ml). Values obtained for derivatized samples were corrected for the same concentration of values obtained for underivatized samples. The separation buffer was 0.05 *M* sodium tetraborate buffer (pH 8.3) containing 0.025 *M* LiCl.

Sample	Migration time (min)		Peak area (arbitrary units)	
	Native	Derivatized	Native	Derivatized
L-Arginine	7.48	15.09	1 184 351	106 059 100
Anti-TAC	10.93	23.37	3 192 314	7 041 242
N-Acetyltryptophan	19.58	19.51	1 548 623	1 518 171

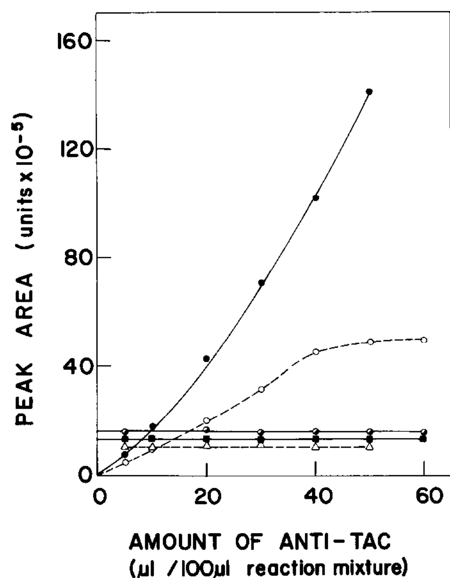


Fig. 3. Calibration graph for anti-TAC monoclonal antibody. The reaction was carried out in the presence of fixed concentrations of internal standards and increasing concentrations of bulk anti-TAC. Typical response curves of (○) native (underivatized) anti-TAC monoclonal antibody, (●) fluorescamine-derivatized anti-TAC, (◑) derivatized and underivatized N-acetyltyptophan, (■) underivatized L-arginine and (△) derivatized L-arginine. The concentrations of analytes used, per 100 μl of reaction mixture, were as in Fig. 2.

each found to have an effect on the separation and quantification of the anti-TAC monoclonal antibody. Under conditions of constant voltage (29 kV), constant temperature (25°C) and constant current (less than 2% drop from the starting current), excellent reproducibility was obtained for peak areas (Table II). The running buffer containing the additive TMAPS, however, seems to yield results with better reproducibility than buffers containing LiCl or TMAC. In addition, significantly less current was produced with the TMAPS buffer than with the buffers containing either of the salt additives. Apparently, the lower operating current with the TMAPS buffer results in the generation of less internal column heat and as a result a slightly higher degree of reproducibility.

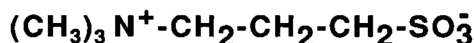
As with the LiCl-containing buffer (Fig. 2), a linear response curve for the fluorescamine-derivatized anti-TAC as a function of concentration was observed with the TMAC and TMAPS buffer systems (Fig. 5). In contrast to the behavior with the LiCl-containing buffer, however, the underivatized analyte did not reach an absorption plateau at higher concentrations, further supporting the hypothesis that the LiCl is adversely effecting analyte solubility.

Optimization of the buffer constituent concentrations seems to be critical for the analysis of quantitative data. Anti-TAC was separated to completion when using 0.05 *M* sodium tetraborate (pH 8.3) (Fig. 6A), but the peak area was slightly lower than when using the same buffer in the presence of 0.025 *M* TMAPS (Fig. 6B). Further, if the concentration of TMAPS was increased to 0.5 *M*, all analytes migrated faster, to a point at which both resolution and quantification were poorer, *e.g.* when 1.0 *M* TMAPS was used as additive to the sodium tetraborate buffer (results not shown).

The presence of internal standards was useful in monitoring the performance of the CE system. Disturbances in the electroosmotic flow, which might result in changes in observed peak areas and lead to errors in quantification, would be reflected in changes in the internal standard controls.

An interesting observation was made with regard to the migration and detection of analytes in a mixture of substances. As shown in Fig. 6, the anti-TAC monoclonal antibody (peak 5) migrated much faster than the fluorescamine reagent (peak 6). Nevertheless, the width of the anti-TAC peak was much greater than that of the fluorescamine reagent. Hence, in addition to mobility, and the corresponding residence time in the optical path of the detector, analyte peak width must also be a function of other factors. With anti-TAC, for example, such factors might include capillary wall interaction, shape of the molecule and solubility.

TETRAMETHYLAMMONIUM CHLORIDE



TRIMETHYLAMMONIUM PROPYLSULFONATE

Fig. 4. Schematic representation of the molecular structures of tetramethylammonium chloride and trimethylammonium propylsulfonate.

TABLE II

EFFECT OF BUFFER CONSTITUENTS ON THE DETERMINATION (PEAK AREA) OF FLUORESCAMINE-DERIVATIZED HUMANIZED ANTI-TAC MONOCLONAL ANTIBODY AND INTERNAL STANDARDS

The composition of the buffers was as follows: 0.05 *M* sodium tetraborate (pH 8.3), (A) contaning no additives; (B) containing 0.025 *M* LiCl; (C) containing 0.025 *M* TMAC; and (D) containing 0.025 *M* TMAPS. For L-arginine and N-acetyltryptophan experiments, the mixture consisted of fixed concentrations of L-arginine (10 μl of a 1 mg/ml solution) and N-acetyltryptophan (10 μl of a 1 mg/ml solution) and increasing concentrations of bulk anti-TAC (5, 10, 20, 30, 40 and 50 μl of a 6.7 mg/ml solution). For the anti-TAC experiment, a fixed concentration of all three analytes was used: anti-TAC (40 μl of a 6.7 mg/ml solution), L-arginine (10 μl of a 1 mg/ml solution) and N-acetyltryptophan (10 μl of a 1 mg/ml solution). For the three experiments, sample volumes were adjusted to 70 μl with 0.1 *M* sodium tetraborate buffer (pH 9.0) and then 30 μl of fluorescamine reagent were added to the sample as described under Experimental.

Buffer	Parameter[a]	Anti-TAC	L-Arginine	N-Acetyltryptophan
A	n	6	6	6
	$\bar{x}$	10 854 734	1 026 934	1 491 201
	S.D.	398 723	25 865	38 976
	R.S.D. (%)	3.67	2.52	2.61
B	n	6	6	6
	$\bar{x}$	10 972 325	1 043 421	1 501 511
	S.D.	364 719	25 635	38 688
	R.S.D. (%)	3.32	2.46	2.58
C	n	6	6	6
	$\bar{x}$	13 324 256	1 218 024	1 585 017
	S.D.	362 654	28 001	31 820
	R.S.D. (%)	2.72	2.30	2.00
D	n	6	6	6
	$\bar{x}$	13 009 167	1 024 243	1 369 606
	S.D.	328 116	22 419	26 566
	R.S.D. (%)	2.52	2.19	1.94

[a] $\bar{x}$ = Mean peak area (arbitrary units); S.D. = standard deviation; R.S.D. = relative standard deviation.

DISCUSSION

Therapeutic monoclonal antibodies and recombinant proteins in general are gaining great importance in the new generation of drugs targeted for human and animal consumption [33]. The non-human origin of these materials, however, and the rigorous purification procedure to which they may be subjected, make proper quality control of the final product and the demonstration of an extremely high degree of purity essential. Many analytical techniques are routinely required for the monitoring of the purity and stability of antibodies. CE is growing continously in the scope of its applications and holds the promise of becoming a routine method for the analysis of proteins. However, in order for any analytical technique to be useful, the results obtained must be reproducible. For CE, reproducibility of separation (migration time) is commonly obtained with a relative standard deviation (R.S.D.) of less than 1% for most tested substances. Nevertheless, quantification of the analytes (peak area) varies, for macromolecules such as proteins, from 1 to 5% (R.S.D.).

As therapeutic monoclonal antibodies are routinely produced by methods involving extensive purification schemes, many factors must be evaluated in order to guarantee a product of consistant quality. These laboratory-made proteins are designed to be similar to, if not identical with, their endogenous counterparts. Therefore, not only must the protein be chemically pure, it must also maintain a structural integrity, *i.e.*, conformation necessary for biological activity and the maintenance of the native state.

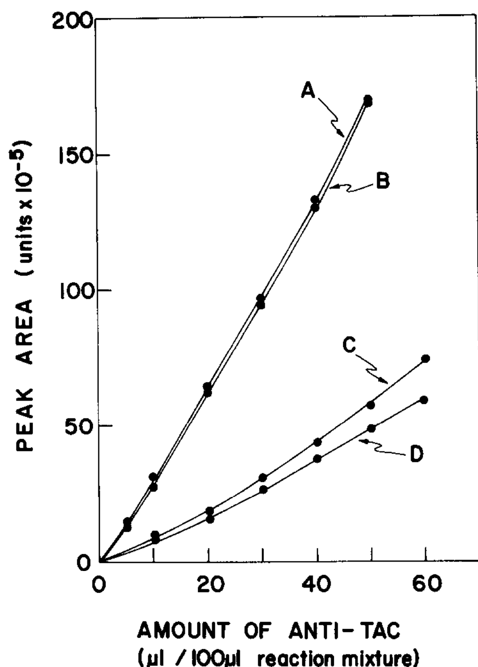


Fig. 5. Comparative calibration graphs for anti-TAC monoclonal antibody using 0.05 *M* sodium tetraborate (pH 8.3) as the separation buffer containing either TMAC or TMAPS. The reaction was carried out in the presence of fixed concentrations of internal standards and increasing concentrations of bulk anti-TAC solution. Typical response curves of (A) fluorescamine-derivatized anti-TAC in the presence of 0.025 *M* TMAC; (B) fluorescamine-derivatized anti-TAC in the presence of TMAPS; (C) native (underivatized) anti-TAC in the presence of TMAC; and (D) underivatized anti-TAC in the presence of TMAPS.

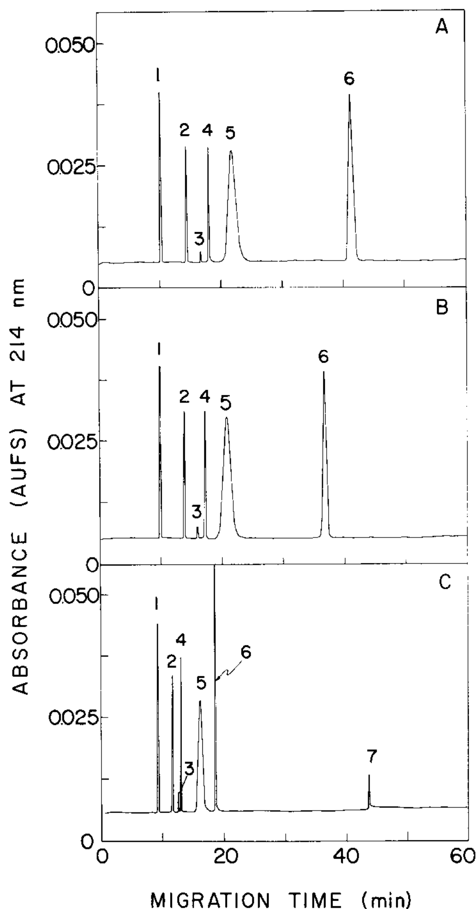


Fig. 6. Capillary electrophoresis profile of fluorescamine-derivatized anti-TAC separated under three different buffer conditions: (A) 0.05 *M* sodium tetraborate buffer, (pH 8.3); (B) 0.05 *M* sodium tetraborate buffer (pH 8.3) containing 0.025 *M* TMAPS; and (C) 0.05 *M* sodium tetraborate buffer (pH 8.3) containing 0.5 *M* TMAPS. The humanized anti-TAC monoclonal antibody used in this experiment was present in a simple liquid formulation dosage form at a concentration of 40 μl (240 μg or 1.60 pmol) per 100 μl of reaction mixture, containing 0.2 mg/ml of Tween-80. Peaks as in Fig. 2, except that peak 7 represents an unknown substance.

The proper interpretation of data necessitates that precautions be taken in designing the technical aspects of the analysis. CE has many operational factors that must be considered in order to obtain consistant reproducibility values for migration time and peak area. At pH $>$ 3.0, the surface of the fused-silica capillary column is negatively charged and proteins with a strong positive charge (basic proteins) have a greater tendency to adhere to the walls of the capillary column. As a consequence, separation and recovery are poor and often the adsorption is irreversible.

The experiments described here demonstrate the importance of blocking the negative charges in order to improve separation and quantification. Lithium chloride, tetramethylammonium chloride and trimethylammonium propylsulfonate as running buffer constituents proved to be effective substances, when used in combination with moderate ionic strength buffers, for preventing adsorption of the monoclonal antibody to the capillary wall.

Apparently, both hydrophobic and electrostatic forces are involved in the adsorption of proteins to glass surfaces. There is a significant coulombic attraction of positively charged regions of the protein to the capillary surface. Some proteins have more

affinity to silicic acid groups than others because of the nature and structural conformation of the molecule. The protein-adsorption blocking power of some buffer constituents (*e.g.*, tetraalkylamines) can be attributed to their dual character as hydrophobic electrolytes as the molecules have both apolar and polar properties. Zwitterions, having both a positive and a negative charge in the molecule, can also compete for the groups which attract proteins to their surfaces and, in turn, prevent adsorption of the proteins to the capillary walls. Adsorption is only one of the factors that may affect the quantitative precision and accuracy of open-tubular free-solution CE [34]. It is possible that alternative approaches, such as the use of gel-filled capillaries, will minimize some sources of CE variability (*e.g.*, diffusion) that may lead to quantification errors. Many practical problems, however, remain to be solved.

CONCLUSIONS

Resolution, recovery and reproducibility for proteins separated by CE are strongly compromised when adsorption of proteins to fused-silica capillaries occurs. Addition of alkylamines and/or zwitterions improves the performance of separation and enhances the efficiency, resolution and reproducibility for protein analytes. In addition, the formation of fluorescamine derivatives significantly affects the separation and enhances the sensitivity for therapeutic proteins.

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