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Assay of protein drug substances present in solution mixtures by fluorescamine derivatization and capillary electrophoresis

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ABSTRACT

A method is described to enhance the resolution and detection sensitivity of proteins, peptides, and amino acids in capillary electrophoretic analysis of solution mixtures. The method consists of derivatizing the analytes with fluorescamine, which is normally used as a fluorogenic reagent for compounds containing a reactive primary amine functional group, and then using the derivative as an ultraviolet chromophore to enhance detection sensitivity (measured at 280 nm) in capillary electrophoresis. The results demonstrated a significant improvement in the separation and detection sensitivity of the derivatized analytes as compared to their underivatized counterparts. The use of chromophores, such as fluorescamine, in capillary electrophoresis facilitates the analysis of components of solution mixtures, such as pharmaceutical formulations, that could not be resolved and/or detected by conventional capillary electrophoresis procedures.

INTRODUCTION

The utility of capillary electrophoresis (CE) for the analysis of protein drug substances present in a solution mixture has been previously demonstrated [1,2]. Because of the complexity of many solution mixtures, however, it is essential to develop CE conditions that will provide a sufficient degree of resolution to separate the components of the analyte mixture. A typical complex solution mixture, for example, is a pharmaceutical formulation. In this case, in addition to optimization of separation conditions, detection sensitivity must be maximized in order to visualize small amounts of drug substances in the presence of relatively large amounts of excipients. With this in mind, we have developed a method for increasing the detection sensitivity in the CE analysis of protein drug substances. The method involves reaction of the analyte mixture with the reagent fluorescamine to form fairly stable derivatives. These derivatives can then be detected with a conventional UV detector. The fluorescamine-analyte derivatives are separated with a higher degree of resolution than their underivatized counterparts.

Fluorescamine (4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione) has been commonly used as a fluorogenic reagent [3–9]. This reagent reacts readily and rapidly at alkaline pH with primary amines to form intensely fluorescent substances, providing the basis for a rapid and sensitive assay of amino acids, peptides, proteins, and other primary amines [7–12]. Although free fluorescamine is quickly hydrolyzed in aqueous solution, the derivative it forms with primary amines is not. The comparative rates of the hydrolysis and derivatization reactions are crucial parameters. At pH 9 and ambient temperature, the reaction between fluorescamine and primary amines has a half-time of 200–1000 ms, while the hydrolytic reaction has a half-time of several seconds [7]. This assures that, upon mixing fluorescamine with an aqueous solution which con-

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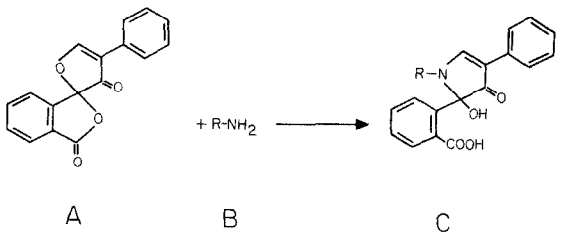

Fig. 1. Schematic representation of the molecular structure of fluorescamine (A), the reacting amino functional group-containing analyte (B) and the derivatized reaction product (C).

tains primary amines, there will be immediate derivatization followed by rapid hydrolysis of unreacted reagent. The proposed reaction scheme of fluorescamine with primary amines [7] is shown in Fig. 1.

The vast amount of data available in the literature has focused on the use of fluorescamine as a fluorogenic reagent (assaying for fluorescent derivatives). Only a few papers [13–16] report the measurement of the fluorescamine-analyte derivative by UV absorption. In this report we have examined the utility of fluorescamine as a UV chromophore, and have evaluated its application to the measurement of protein drug substances in pharmaceutical formulations separated by capillary electrophoresis. As a model system we have used the active drugs recombinant human leukocyte A interferon[a], humanized anti-TAC monoclonal antibody[b] and the commonly used excipients glycine, L-arginine and human serum albumin.

EXPERIMENTAL

Reagents and supplies

All chemicals were obtained at the highest purity level available from the manufacturer, and were used without additional purification. Potassium hydroxide, sodium phosphate (NA_2HPO_4), borax ($Na_2B_4O_7 \cdot 10H_2O$), lithium chloride, crystallized human serum albumin, and fluorescamine were obtained from Sigma (St. Louis, MO, USA). Glycine and L-arginine were purchased from Fluka (Ronkonkoma, NY, USA). Acetone (HPLC grade), pyridine (Fisher Certified), and hydrochloric acid solution (12 *M*) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Recombinant human leukocyte-A interferon and humanized anti-TAC monoclonal antibody were supplied by Hoffmann-La Roche (Nutley, NJ, USA). Albuminar-25 (250 mg/ml solution for injection of human serum albumin) was purchased from Armour Pharmaceutical (Kankakee, IL, USA). Reagent solutions and buffers were prepared using triply distilled and 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 were obtained from Scientific Glass Engineering (Austin, TX, USA), and Polymicro Technologies (Phoenix, AZ, USA).

Instrumentation

A commercially available CE instrument (P/ACE System 2000, Beckman, Palo Alto, CA, USA), was used for this work. In this instrument, the capillary is housed in a cartridge constructed so as to allow a flow of recirculating liquid for Peltier-temperature control of the capillary column. 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 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 into the sample compartment of the CE instrument. Samples were injected into the capillary column by pressure. Peak visualization and data acquisition were performed using the UV detection system of the CE instrument and the System Gold chromatography software package (Beckman, San Ramon, CA, USA). Data integration was also carried out with a Model D-2500 Chromato-Integrator (Hitachi, Danbury, CT, USA).

[a] Leukocyte A interferon (IFN-αA) is in current nomenclature designated IFN.α2a.

[b] Anti-TAC (T-activated cell) is an IgG1 class genetically engineered hybrid antibody, directed against the human receptor for interleukin-2 [26].

Assay procedure

Sample preparation. Equal molarity stock solutions were individually prepared by dissolving L-arginine (1 mg/ml) and glycine (0.43 mg/ml) in 0.1 *M* sodium tetraborate (borax) buffer, pH 9.0. A stock solution of crystallized human serum albumin (25 mg/ml), used as reference, was prepared in the same buffer. Albuminar-25 (250 mg/ml), recombinant human leukocyte-A interferon (5.8 mg/ml) and humanized anti-TAC monoclonal antibody (8.6 mg/ml) were obtained as concentrated solution and 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 desired concentrations with sample dilution buffer (0.1 *M* sodium tetraborate buffer, pH 9.0) and directly transferred to the conical vial and then inserted into the microapplication vessel assembly on the CE instrument.

For CE analysis of fluorescamine derivatives, solutions of the respective analyte samples (concentration ranging from 2.1 to 1250 μg, or from 7.4 to 172.2 nmol per 100 μl reaction mixture) were transferred to a 500-μl microcentrifuge tube and their total volume 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 fluorescamine in acetone, containing 20 μl pyridine) to the sample while continously and vigorously vortexing. After approximately 2 min, the content of the microcentrifuge tube was transferred to the conical microvial and then inserted into the microapplication vessel assembly for analysis.

For the fluorescamine-derivatized L-arginine the volumes of the sample and reagent were doubled and prepared in a 500-μl microcentrifuge tube. A 100-μl aliquot was transferred to a conical microvial and then inserted into the microapplication vessel assembly as described above. The rest of the sample mixture was maintained in a sealed microcentrifuge tube at 25°C, and used as a replacement sample for the last two experimental points (8.5 and 24.5 hours).

Running conditions. Sample solutions for analysis in microapplication vessels were placed into 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 4 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, distilled-deionized water, and then regenerated with running buffer.

The CE separations reported were performed using three different buffers: (1) 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.05 *M* lithium chloride; (2) 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.025 *M* lithium chloride; or (3) 0.05 *M* sodium phosphate buffer, pH 7.0, containing 0.05 *M* lithium chloride. The CE instrument was equipped with a 57 cm (50 cm to the detector) × 75 μm I.D. capillary column. The CE separation was performed at 12 kV when using the 0.05 *M* lithium chloride-containing sodium phosphate buffer, at 15 kV when using the 0.05 *M* lithium chloride-containing sodium tetraborate buffer, or at 18 kV when using the 0.025 *M* lithium chloride-containing sodium tetraborate buffer. Capillary temperature for all experiments was maintained at 25°C during the run. Under these conditions, approximately 24 nl (6 nl/s) was injected into the capillary column [17]. Monitoring of the analytes was performed at wavelengths of 200, 214 or 280 nm.

RESULTS

Fig. 2 depicts the electropherograms of a control fluorescamine solution and of a fluorescamine-derivatized simple mixture of primary amines, *i.e.*, L-arginine and glycine. Both of the fluorescamine-derivatized amino acids, L-arginine (peak 2) and glycine (peak 3), are well separated from each other and from the peaks corresponding to the constituents of the derivatization reagent, fluorescamine (peak 4) and the organic solvents acetone and pyridine (comigrating at peak 1). The observed decreased peak area for the fluorescamine (Fig. 2B, peak 4), in comparison with the control value (Fig. 2A, peak 4), confirms that a significant amount of the reagent is immediately consumed in reaction with the analytes.

In order to optimize the degree of derivatization of the analytes with fluorescamine under the reaction conditions used, *i.e.*, pH 9.0 in a sodium tetraborate buffer at 25°C, a fixed amount of L-arginine (115 nmol) was reacted with increasing concentra-

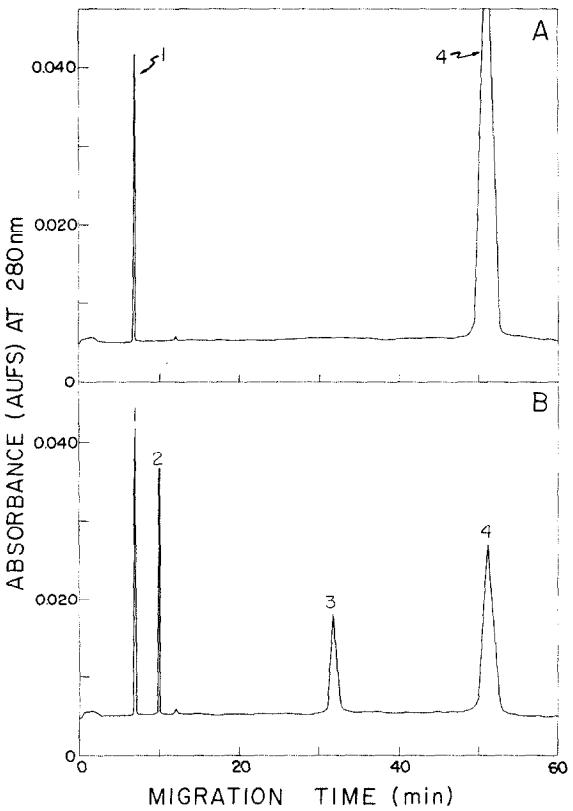

Fig. 2. Capillary electrophoresis profile of fluorescamine-derivatized amino acids. (A) Electropherogram of the fluorescamine solution control; peaks: 1 = acetone; 4 = fluorescamine reagent. (B) Electropherogram of glycine and L-arginine; peaks: 1 = acetone; 2 = fluorescamine-derivatized L-arginine; 3 = fluorescamine-derivatized glycine; 4 = fluorescamine reagent. The procedure was carried out as described in the Experimental section. The separation buffer consisted of 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.05 *M* lithium chloride.

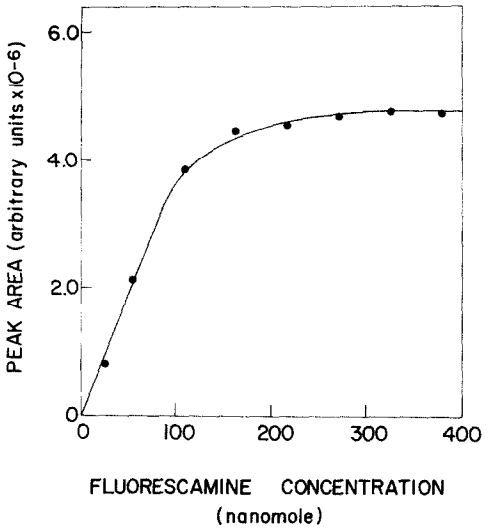

Fig. 3. Relationship between peak area of derivatized analyte formed and concentration of fluorescamine consumed. The amount of excess fluorescamine reagent was calculated by reacting a fixed concentration of L-arginine (115 nmol/100 μl reaction mixture volume) with an increasing concentration of fluorescamine. The procedure was carried out as described in the Experimental section. The separation buffer consisted of 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.05 *M* lithium chloride.

tions of the fluorescamine reagent. As shown in Fig. 3, peak area of the derivative appeared to be maximal at a fluorescamine concentration of 250–300 nmol (per 100 μl reaction mixture volume). Therefore, for this amino acid, a two- to three-fold molar excess of fluorescamine reagent should be sufficient to saturate the reaction mixture and form an optimal fluorescamine-amino acid derivative.

Using these conditions, the linearity of the derivative peak area as a function of L-arginine concentration was investigated. As shown in Table I and Fig. 4, a linear response (at 280 nm) was observed at L-arginine concentrations ranging from 14.3 nmol/100 μl to 172.2 nmol/100 μl of reaction mixture. A similar molar excess fluorescamine ratio and saturation response curve were observed for glycine (data not shown).

The stability of the fluorescamine-L-arginine derivative over a 24-h period, which might constitute a normal day's assay requirements, was also investigated. For this study, caution was taken to avoid evaporation during the 24-h storage period. This is particularly important in view of the volatile nature of the fluorescamine solvent (acetone). As shown in Table II, no loss in derivative was observed over the test period. On the contrary, peak area was observed to increase slightly over the first 2.5 h of storage and then remain constant. The progressive increase in peak area during the initial phase of storage is most likely the result of evaporative concentration, which appears to occur to some extent despite the precautions taken. This is probably due in part to: (a) storage of the sample in the ambient temperature environment of the CE instrumentation sample handling compartment, and (b) poor or

TABLE I

TYPICAL AMOUNTS OF FLUORESCAMINE-DERIVATIZED L-ARGININE ANALYZED BY CAPILLARY ELECTROPHORESIS AT 280 nm

For this experiment, increasing amounts of L-arginine were reacted with a fixed concentration of fluorescamine reagent (324 nmol/100 μl reaction mixture). The separation buffer consisted of 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.05 *M* lithium chloride.

Amount of L-arginine present in 100 μl reaction mixture			Amount of L-arginine injected into the capillary column			
μl	μg	nmol	nl	ng	pmol	peak area (arbitrary units × 10^{-6})
2.5	2.5	14.3	24	0.6	3.4	0.57
5	5	28.7	24	1.2	6.7	1.09
10	10	57.4	24	2.4	13.8	2.20
15	15	86.1	24	3.6	20.7	3.18
20	20	114.8	24	4.8	27.6	4.48
25	25	143.5	24	6.0	34.4	5.59
30	30	172.2	24	7.2	41.3	6.92

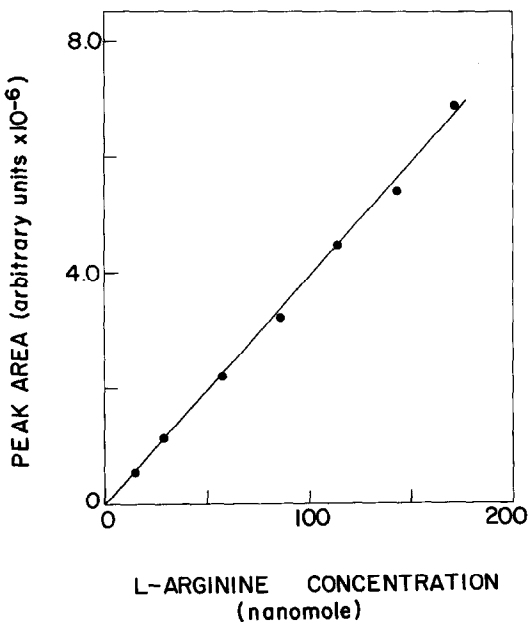

Fig. 4. Relationship between peak area of the derivatized analyte formed and the concentration of reacting L-arginine. The linearity of the formation of fluorescamine-derivatized amino acid was calculated by reacting increasing concentrations of L-arginine (ranging from 14.3 to 172.2 nmol/100 μl reaction mixture) with an excess amount of fluorescamine reagent (324 nmol/100 μl reaction mixture). The procedure was carried out as described in the Experimental section. The separation buffer consisted of 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.05 *M* lithium chloride.

incomplete sealing of the microapplication vessel assembly. Without the precautions taken, *i.e.* humidification of the chamber and water cooling of the sample, evaporation occurred much more rapidly and resulted in a dramatic loss of acetone and a corresponding concentration of the fluorescamine-L-arginine derivative (results not shown). Similarly, evaporative concentration effects have been previously demonstrated for benzoic acid derivatives [18].

TABLE II

STABILITY OF FLUORESCAMINE-L-ARGININE DERIVATIVE

For this experiment, the microapplication sample vessel containing the microvial was filled with an appropriate amount of deionized water in order to avoid evaporation. For details see the Experimental Section. The separation buffer consisted of 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.05 *M* lithium chloride.

Time of incubation (h)	Peak area (arbitrary units × 10^{-6})
0.5	4.58
1.5	4.61
2.5	4.72
4.5	4.78
8.5	4.79
24.5	4.76

The fluorescamine derivatization procedure developed with the simple amino acid model compounds was applied to CE analysis of recombinant human leukocyte A interferon and human serum albumin, which represent a typical protein drug substance and a common protein excipient. For derivatization, fluorescamine concentration was maintained at 324 nmol/100 μl of reaction mixture and analyte samples containing 13.3 nmol of interferon and 4.2 nmol of albumin, respectively, were analyzed. CE analysis was performed using the 0.05 *M* sodium phosphate buffer, pH 7.0, containing 0.05 *M* lithium chloride. As shown in Fig. 5, the derivatization with fluorescamine resulted in a 20- to 30-fold increase in detection response for the protein analytes. Under these conditions (280 nm), interferon at a concentration down to 400 μg/ml (20.4 nmol/ml), and albumin at a concentration down to 800 μg/ml (11.7 nmol/ml) were easily detected when derivatized with fluorescamine.

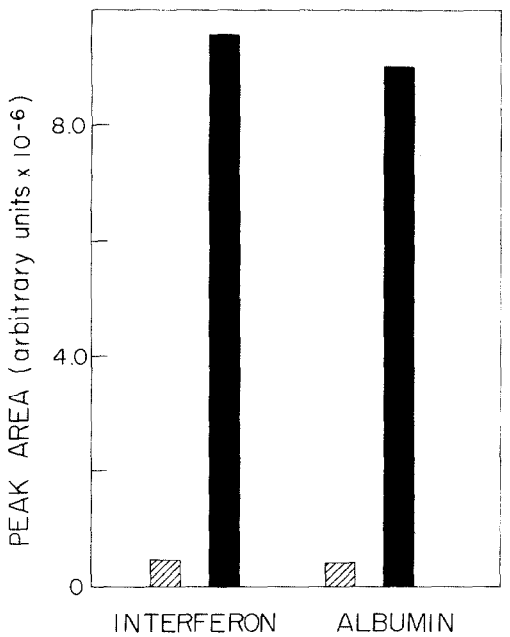

Fig. 5. Comparison of the peak areas of the non-derivatized proteins versus fluorescamine-derivatized human serum albumin and recombinant human leukocyte A interferon. Dashed bars represent the peak areas of non-derivatized proteins. Solid bars represent the peak areas of fluorescamine-derivatized proteins. The concentration of non-derivatized samples used in this experiment was 580 μg (29.6 nmol)/100 μl reaction mixture of recombinant leucocyte A interferon, and 1250 μg (18.4 nmol)/100 μl reaction mixture of human serum albumin. The concentration of fluorescamine-derivatized samples was 290 μg (14.8 nmol)/100 μl reaction mixture of recombinant leucocyte A interferon, and 250 μg (3.7 nmol)/100 μl reaction mixture of human serum albumin. The separation buffer consisted of 0.05 *M* sodium phosphate buffer, pH 8.3, containing 0.05 *M* lithium chloride.

Derivatization with fluorescamine was also observed to have a marked effect on analyte mobility in CE analysis. As indicated in Table III, derivatization increased the migration times for both the amino acid and protein analytes. The magnitude of the shift appears to be mostly determined by the net charge of the analyte after derivative formation, which is a function of both the p*I* of the underivatized analyte and the number of reacting amino groups present. The molar ratio of fluorescamine to analyte in the derivative also influences migration rate, and would play a major role in determinating the mobility of analytes migrating in buffers at or near their p*I* values.

This mobility shift is graphically illustrated in Fig. 6, which shows the electropherograms obtained for leukocyte A interferon samples (2.9 nmol/100 μl, 24 nl injection) assayed with and without fluorescamine derivatization. Analysis of the non-derivatized sample (Fig. 6A) indicated a single peak migrating at 10.2 min and detected near the lower limits of detector sensitivity (at 280 nm). Derivatization with fluorescamine (Fig. 6B) shifted the interferon to 22.2 min (peak 4), increased its peak area dramatically, and enabled detection of an un-

TABLE III

EFFECT OF FLUORESCAMINE ON THE MIGRATION TIME OF COMPONENTS PRESENT IN A FORMULATION MIXTURE

For this experiment, CE detection of separated native amino acids (non-fluorescamine derivatized) was performed at 200 nm. All other measurements were carried out at 280 nm. For the separation of the formulation mixture sample components by CE, 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.025 *M* lithium chloride was used.

Substance	Migration time (min)	
	Native	Derivatized
L-Arginine	3.9	7.3
Glycine	5.2	20.0
Humanized anti-TAC	5.9	10.1
Interferon	7.2	14.0
Human serum albumin	9.7	17.8

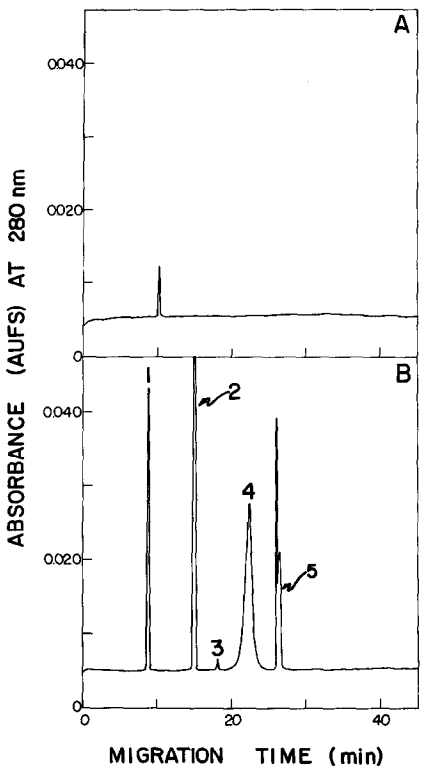

Fig. 6. Capillary electrophoresis profile of recombinant leukocyte A interferon. (A) Electropherogram of non-derivatized interferon. (B) Electropherogram of fluorescamine-derivatized interferon. Peaks: 1 = acetone; 2 = ammonia; 3 = unknown; 4 = interferon; 5 = fluorescamine reagent. The separation buffer consisted of 0.05 *M* sodium phosphate buffer, pH 7.0, containing 0.05 *M* lithium chloride. The concentration of interferon used in this experiment was 580 μg (29.6 nmol)/100 μl reaction mixture (A), and 232 μg (11.8 nmol)/100 μl reaction mixture (B).

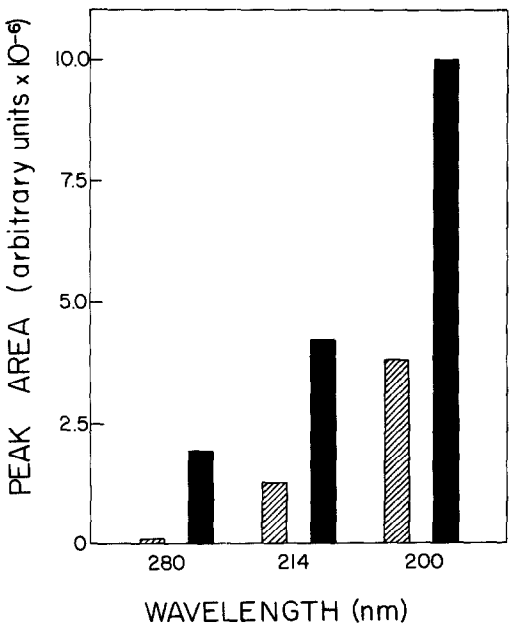

Fig. 7. Comparison of the peak areas of non-derivatized and fluorescamine-derivatized interferon at various wavelengths. Dashed bars represent the peak areas of the non-derivatized interferon and solid bars represent fluorescamine-derivatized interferon. The separation buffer consisted of 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.025 *M* lithium chloride. The concentration of interferon used in this experiment was 58 μg (2.9 nmol)/100 μl reaction mixture.

known component (peak 3) and ammonia (peak 2), an interferon buffer constituent. The enhanced sensitivity afforded by fluorescamine derivatization is even greater when detection is performed at lower wavelengths (Fig. 7).

The procedures developed using the model compounds were applied to CE analysis of recombinant leukocyte A interferon (Fig. 8A) and recombinant humanized anti-TAC antibody (Figure 8B) in formulation mixtures. To demonstrate the versatility of CE, the formulation mixtures were supplemented with the excipients human serum albumin, glycine and L-arginine, which may also serve as reference compounds for mobility comparison. As shown in the figure, all of the compounds were well separated.

DISCUSSION

Proteins and peptides are playing an ever increasing role in todays pharmaceutical industry. Although capillary zone electrophoresis and other variants of CE methods have developed over the last decade into analytical tools of remarkable performance [19–25], the use of this technology for the separation and analysis of proteins is not yet generally accepted. Difficulties in separating the proteins of interest from components of the formulation matrix and the high levels of sensitivity required for their detection, have contributed to the problem. Furthermore, detection of proteins is difficult owing to a lack of good chromophore groups in easily accesible spectral regions.

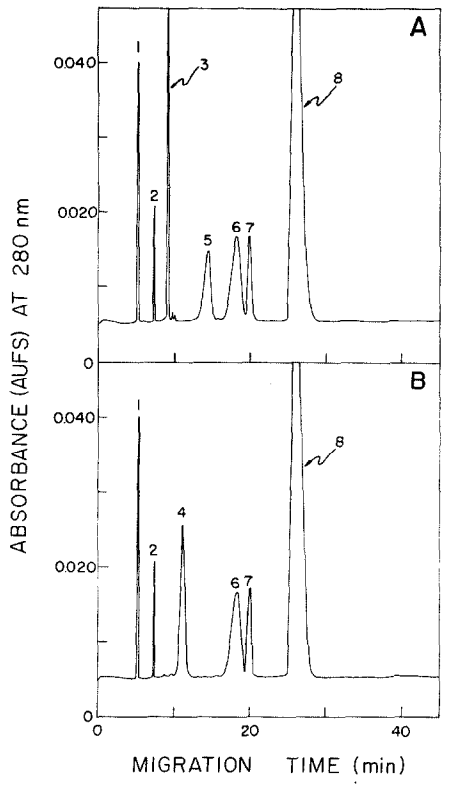

Fig. 8. Capillary electrophoresis profile of fluorescamine-derivatized formulation mixture components. (A) Electropherogram of a formulation mixture containing human leukocyte A interferon; peaks: 1 = acetone; 2 = L-arginine; 3 = ammonia; 5 = interferon; 6 = human serum albumin; 7 = glycine; 8 = fluorescamine reagent. (B) Electropherogram of a formulation mixture containing humanized anti-TAC monoclonal antibody; peaks: 4 = anti-TAC monoclonal antibody; all other peaks as in (A). The separation buffer consisted of 0.05 *M* sodium tetraborate buffer, pH 8.3, containing 0.025 *M* lithium chloride. The concentration of analytes, per 100 μl reaction mixture, used in this experiment were as follows: L-arginine, 5 μg (28.7 nmol); anti-TAC monoclonal antibody, 215 μg (1.4 nmol); interferon, 145 μg (7.4 nmol); glycine, 2.1 μg (28.7 nmol); and human serum albumin, 125 μg (1.8 nmol).

The experiments described in this report demonstrate that the addition of a chromophore, such as fluorescamine, to an amino functional group of a protein, peptide, or amino acid, or any other amine-containing substance, dramatically improves its detection sensitivity in the UV region. Furthermore, derivatization with fluorescamine shifts analyte migration time, as a function of analyte structure and buffer conditions, and may result in an enhanced degree of resolution as compared to underivatized counterparts. For maximum utility, conditions for derivatization with fluorescamine should be optimized for the analyte of interest. For simple compounds, containing only a single reactive amino group, a 2- to 3-fold molar excess of reagent and a reaction pH of 9.0 were sufficient to show a dramatic increase in detection sensitivity when monitored at 280 nm. For more complex analytes, having many reactive groups, an increased molar ratio of fluorescamine and slightly altered reaction pH, may be called for. Also, it may be possible that the extent of derivatization could be dependent on the sample matrix. Thus, if this problem occurs, post-column derivatization may be used as an alternative method.

Optimization of CE running conditions, to consider the effects of buffer salt, pH and the inclusion of inorganic ions [24,25], is also essential in order to assure the required degree of analyte resolution. For example, the experiments described in this report demonstrated that for the analysis of the fluorescamine-derivatized protein analytes by CE, 50 m*M* lithium chloride-containing sodium tetraborate buffer, pH 8.3, produces adequate separation with reproducible migration times and peak areas. Similar results are obtained with 50 m*M* lithium chloride-containing sodium phosphate buffer, pH 7.0. However, when the analysis of all formulation mixture components is carried out simultaneously, the separation seems to be better when the concentration of lithium chloride is reduced to 25 m*M* in the sodium tetraborate buffer, pH 8.3.

In conclusion, the incorporation of fluorescamine derivatization in the CE analysis of amino acids, peptides or proteins, or of molecules in general containing a reactive primary amine functional group, significantly improves detection sensitivity and separation. The method is simple, quite fast, and very reproducible. The derivatives formed also appear to be stable and show no loss in UV response over the period tested.

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