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Journal of Chromatography A, 1044 (2004) 317–322
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Separation of polyethylene glycol-modified proteins by open tubular capillary electrochromatography

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Available online 19 May 2004

Abstract

This study involves the characterization of six polyethylene glycol-modified proteins by open tubular capillary electrochromatography, a high-resolution, versatile and reproducible technique for the analysis of biomolecules and pharmaceuticals. Optimized conditions were obtained with respect to type of capillary modification (cholesterol and octadecyl), applied voltage (+20 and −20 kV), buffer pH (2.14–8.14) and addition of methanol modifier to the mobile phase. Electrochromatograms were obtained with both cathodic and anodic applied electric fields. In the case of one PEG–protein, superoxide dismutase, a comparison was made to a previous study. Reproducibility and column lifetime were also evaluated in assessing the usefulness of the method.

Keywords: Electrochromatography; Proteins; Enzymes

1. Introduction

Polyethylene glycol (PEG) has been used to chemically modify therapeutic proteins and peptides in order to increase solubility with respect to transport within the body, to provide increased resistance toward proteolytic degradation, to lower toxicity, to decrease immunogenicity and lower the dosage rate of drugs due to a longer residence time in the body [1–3]. It has been demonstrated that site-specific attachment of a PEG moiety does not cause significant loss of biological activity for these therapeutic agents [4,5]. Polyethylene glylated (PEGylated) proteins have been developed for the treatment of hepatitis C [6,7] as well as acute lymphoblastic leukemia and large cell lymphoma [8]. Analysis of PEGylated proteins and peptides for clinical and quality assurance purposes is complicated by the heterogeneity that results from the distribution of the PEG among a number of different sites on the biopolymer [9]. Coupling polyethylene glycol to a peptide or protein involves activating PEG to be suitable for bonding with specific functional groups [1,10]. The most common target for attachment of PEG has been lysine and N-terminal amino groups. The actual compound used in these modifications is monofunctional methoxy-PEG (m-PEG) since this polymer does not crosslink or aggregate. The potential number of species created by attachment of PEG, and hence the number that must be identified in any analysis is given by the following formula [11]:

$$P = \frac{N!}{(N-k)!k!} \quad (1)$$

where N is the number of possible sites and k the number of sites actually modified. This equation assumes that all of the proteins are modified with the same number of PEG chains which may not always be true.

Size-exclusion chromatography has been used to separate PEGylated proteins from the native species but the resolving power is insufficient to separate the positional isomers of the modified molecules [12–14]. Hydrophobic interaction chromatography, reversed-phase HPLC and cation exchange chromatography have all had limited success in fully resolving these complex samples [5,12–14]. In each case, the method is complicated by tedious and time-consuming sample preparation steps. Another approach tested has been the combined use of capillary electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In the analysis of PEG–superoxide dismutase (SOD), both the CE and MS data agreed reasonable well with the number of species

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doi:10.1016/j.chroma.2004.03.082

present [15]. In this case, only eight isomers were found in the PEG–SOD sample. In the analysis of digested PEGylated salmon calcitonin, the CE electropherogram displayed only the three major fragments produced [16]. However, the MALDI-TOF-MS data indicated a more complex make-up for each of the fragments representing the polydispersity of the PEG modification.

A promising solution to resolving complex mixtures like PEGylated proteins and peptides is open tubular capillary electrochromatograhy (OT-CEC). This hybrid technique combines the separation mechanisms found in both chromatographic (solute/bonded phase interactions) and electrophoretic (differences in electrophoretic mobility) methods. It retains most of the high efficiency of capillary electrophoreis and provides the selectivity achieved with a chromatographic stationary phase [17]. In the open tubular approach the stationary phase is attached to the wall of a fused silica capillary and solutes are moved through the system by the electrical forces (electroosmotic flow and electrophoretic mobility) created when a high voltage is applied to the electrolyte. Chromatographic interactions are absent if an ordinary fused silica capillary is used. In the method utilized in this study the inner surface of the capillary is etched at high temperature resulting in a surface area increase of 1000-fold and radial extensions that decrease the distance a solute needs to travel to interact with a stationary phase [18]. After etching, the inner wall of the capillary is modified by the silanization/hydrosilation method [19–21] that produces a hydride surface (virtually no silanols) and the attachment of an organic moiety via a stable silicon–carbon bond. These alterations to the fused silica capillary wall create a surface that is more biocompatible than unetched or unmodified capillaries. The versatility, usefulness, ruggedness and high resolving power of the etched chemically modified capillaries for OT-CEC has been demonstrated in a number of publications investigating the analysis of synthetic peptides [22–25]. A similar approach was used for the characterization of several commercially available m-PEG proteins in this study.

2. Experimental

2.1. Materials

The fused silica capillary used was 50 μm i.d. from Polymicro Technologies, Phoenix, AZ, USA. Capillary dimensions: cholesterol capillary, total length = 34.5 cm and distance to detection window = 26 cm; C_{18} capillary, total length = 34.0 cm and distance to the detection window = 26 cm.

Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ammonium bifluoride, the etching agent, was purchased from Sigma–Aldrich (St. Louis, MO/Milwaukee, WI, USA), triethoxysilane (Huls America, PA, USA), 1-octadecene, cholesteryl-10-undeceneoate, and hexachloroplatinic acid (Sigma–Aldrich) were used for the modification of the inner walls of the capillary. The buffer materials were as follows: Tris, 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), L-histidine, γ-aminobutyric acid (GABA) and citric acid were purchased from Sigma–Aldrich; boric acid and glacial acetic acid were purchased from American Scientific Products (CA, USA); and imidazole from Calbiochem (San Diego, CA, USA). The following m-PEG proteins (PEG, $M_r \approx 5000$) were obtained from Sigma–Aldrich: PEG–catalase; PEG–asparaginase; PEG–protease; PEG–chymotrypsin; PEG–superoxide dismutase (SOD); and PEG–lipase.

2.2. Instrumentation

Two high-performance capillary electrochromatography (HPCE) instruments were used in this study. One was an Applied Biosystems Model 270-A-HT capillary electrophoresis system (Foster City, CA, USA) equipped with a UV detector. The other HPCE instrument used was an Agilent (Waldbronn, Germany) 3D capillary electrophoresis instrument having a UV detector. The oven used for etching of capillaries was part of a Hewlett-Packard Model 5890 gas chromatograph. The GC oven was used for the control of the temperature and modified such that multiple capillaries could be accommodated.

2.3. CEC experiments

The etched chemically modified capillaries were prepared as described previously [18,26] and can also be obtained from MicroSolv (Long Branch, NJ). Briefly, the fused silica surface was etched with a 5% ammonium bifluoride–methanol solution for a total of 4 h at elevated temperature (300–400 °C) in a gas chromatographic oven. A silica hydride layer was then covalently attached by reacting the etched surface with ~18% (v/v) triethoxysilane solution in dioxane with a hydrochloric acid catalyst (115 μmol). The cholesterol and C_{18} moieties were attached to the hydride using hexachloroplatinic acid as a catalyst. 1-Octadecene or cholesteryl-10-undecenoate was added to 2.0 mL of toluene and 100 μL of catalyst and heated to 70° for 1 h. The solution was then passed through the capillaries, and the capillaries were heated at 100 °C for 24 h. Additional solution was passed through the capillaries each day for 5 days with the capillaries being stored in the GC oven at 100 °C. At the end of the process the capillaries were rinsed with toluene and methanol.

The PEGylated protein sample was prepared by dissolving 1mg of protein in 1 mL of buffer. The following buffer compositions (diluted 1:10) and pH values were used in this study: pH 2.14, 0.3 mol/L H_3PO_4 and 0.19 mol/L Tris [tris(hydroxymethyl)aminomethane]; pH 3.00, 0.3 mol/L citric acid and 0.25 mol/L β-alanine; pH 4.41, 0.3 mol/L acetic acid and 0.375 mol/L γ-aminobutyric

acid; pH 6.00, 0.3 mol/L MES and 0.21 mol/L L-histidine (1-α-amino-β-imidazole propionic acid); pH 7.06, 0.3 mol/L MOPS and 0.215 mol/L imidazole; pH 8.14, 0.1 mol/L Tris and 0.15 mol/L boric acid. Injection was done either electrokinetically at 5 kV or by vacuum. Detection was at 210 nm.

3. Results and discussion

The number of PEGs per mole of protein and the approximate isoelectric point (p*I*) of the unmodified protein (without PEG) are listed in the Table 1. Therefore, the number of peaks that might be expected in the electrochromatogram as predicted by the equation above is substantial depending on both the "*N*" value listed in the table and "*k*", the number of sites actually modified.

Fig. 1 shows some representative results of three of the PEG proteins obtained on the etched capillary chemically modified with a cholesterol moiety on the inner surface. In each case, there are a substantial number components in these samples indicating extensive PEGylation of these proteins, particularly catalase and protease. The resolution, time of analysis, peak shape (A_S is 1.1 or less) and efficiencies (*N* generally in the range of 100,000–200,000) found in these columns is excellent compared to the other separation methods currently available. With respect to MALDI-TOF-MS, the analysis is simpler and less costly. Two of these PEG proteins (catalase and chymotrypsin) were also run on a bare fused silica capillary column. The results are shown in Fig. 2. Even at the relatively low pH value of this analysis no detectable peaks and only random noise spikes are obtained in the electrochropherograms most likely indicating relatively strong or irreversible adsorption of the PEG protein species. After several injections, one or more broad (several minutes in width) perturbations on the baseline are often observed. None of the data on the bare capillary are reproducible while each of the electrochromatograms shown in Fig. 1 are reproducible over five or more consecutive injections as well as after different PEG–protein samples have been tested and the original analyte is injected again.

In order to verify that there is a chromatographic component to the separation of PEGylated proteins by OT-CEC with the etched capillaries, a column with a different organic moiety (C_{18}, octadecyl) was tested on these proteins. Fig. 3 illustrates several aspects of the open tubular, etched chemically modified capillary technology. The sample in this series of electrochromatograms is PEG–protease. It can

Table 1
PEGylated proteins used in this study, the number of moles of PEG potentially attached, and the p*I* of the native protein without the PEG

PEGylated protein	PEGs per mole of protein	~p*I* of unmodified protein
Catalase	40	5.4
Asparaginase	40	4.8
α-Chymotrypsin	9	8.5
Lipase	16	6.1
Superoxide dismutase	12	5.6
Protease	6	8.8

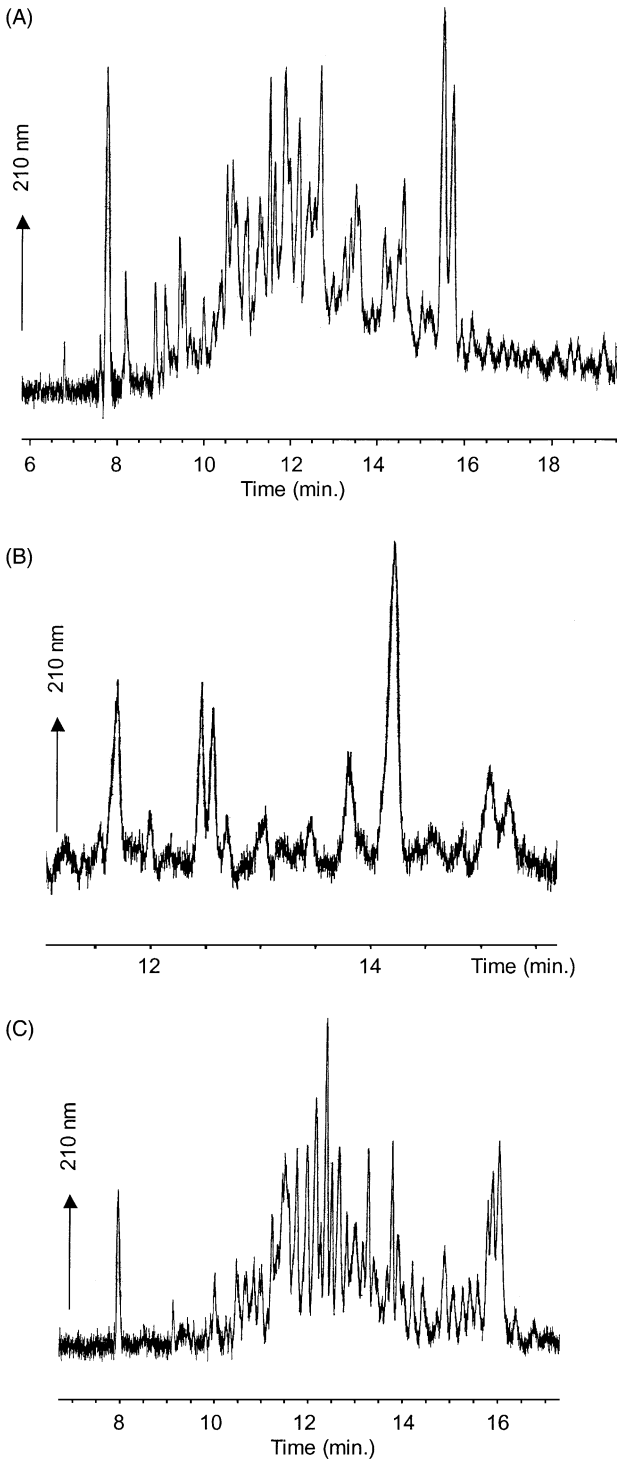

Fig. 1. Electrochromatograms of PEG–proteins on an etched cholesterol modified capillary at 20 kV and pH 2.14: (A) catalase; (B) chymotrypsin and (C) protease.

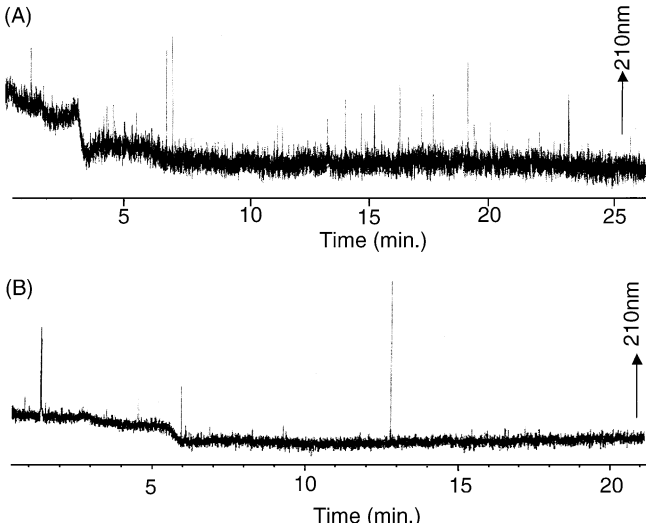

Fig. 2. Electropherograms of PEG–proteins on a bare fused silica capillary: (A) catalase and (B) chrymotrypsin. Experimental conditions same as Fig. 1.

be compared to the electrochromatogram obtained for the same sample using the etched cholesterol capillary shown in Fig. 1C. While the number of peaks is similar (28 for cholesterol and 25 for C_{18}) the elution pattern on the two columns is clearly different. Since the data was obtained under identical experimental conditions (injection time, buffer composition, pH and applied voltage), the electrophoretic migration of the various PEG–proteins should be the same. Only a difference in the degree of interaction between the solutes and bonded material on the inner wall of the capillary would account for a difference in the migration patterns of the species in the sample on the two columns. This data along with numerous previous examples [18,22–30] support the concept of a chromatographic component in protein and peptide separations with etched chemically modified capillaries. Another point is that the injection time in Fig. 3A and B is 5 s while in Fig. 3C and D it is 10 s. Some minor differences are seen in the elution pattern as expected under different injection conditions. Reproducibility for these separations is also excellent since each pair of analyses in Fig. 3 are not consecutive runs ((A) is injection 34 and (B) injection 41; (C) injection 52 and (D) injection 58).

Another interesting feature of the PEG electrochromatograms is illustrated in Fig. 4 for PEG–protease using an applied voltage of −20 kV. A unique feature of the etched chemically modified capillaries is that they display anodic electroosmotic flow (EOF) at pH values lower than $\cong$ 4.5 [26,27]. Therefore, those proteins where the k value in Eq. (1) is high with respect to N, the charge on the protein is very low, perhaps even neutral. As PEGylation

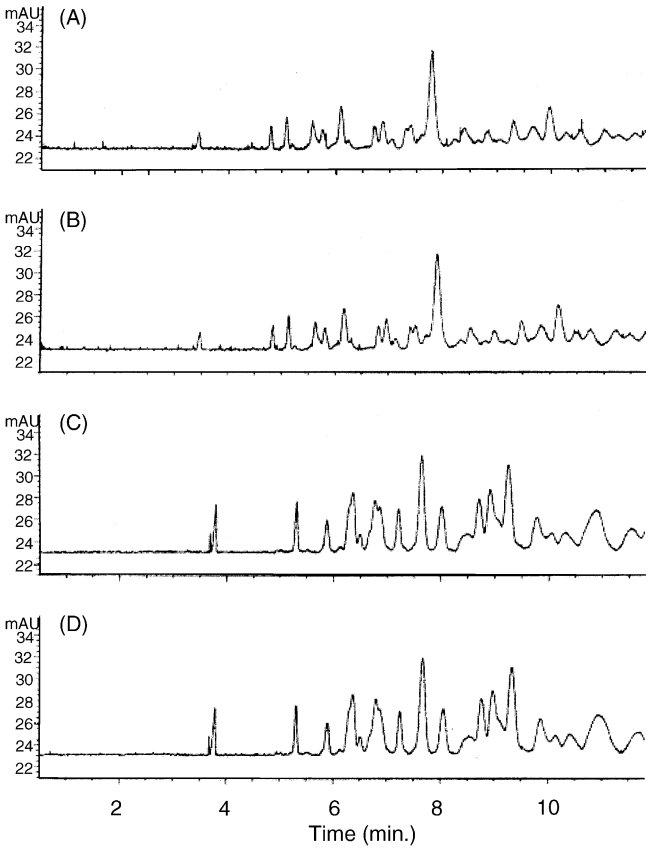

Fig. 3. Electrochromatograms of PEG–protease on an etched C_{18} modified capillary at 20 kV and pH 2.14: (A) and (B) 5 s electrokinetic injection; (C) and (D) 10 s electrokinetic injection.

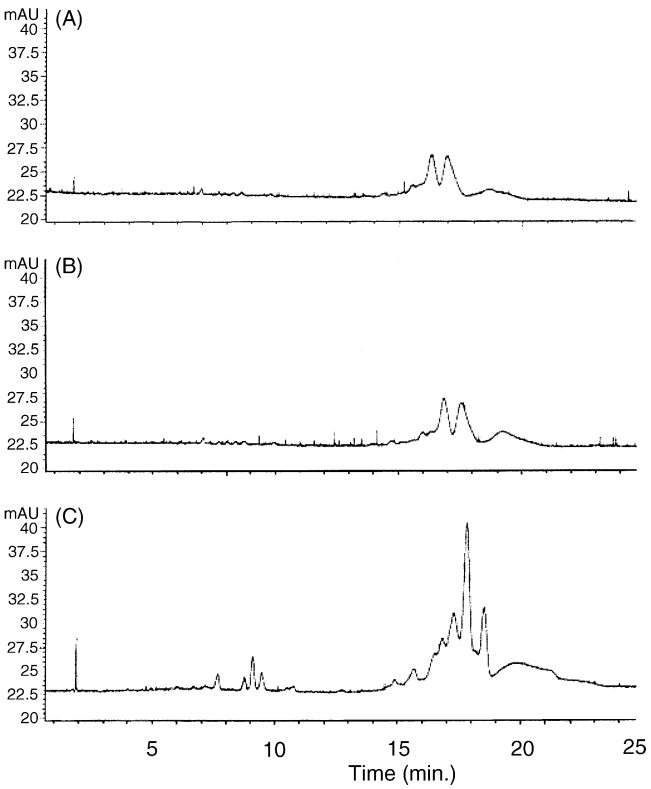

Fig. 4. Electrochromatograms of PEG–protease on an etched C_{18} modified capillary at −20 kV and pH 2.14: (A) and (B) with 5 s electrokinetic injection and (C) with 10 s electrokinetic injection.

removes many sites for protonation on the protein and hence lowers the charge, net migration for some species may be toward the anode and not the cathode. In this case, the net migration of some PEG–protease species is toward the anode as demonstrated in Fig. 4. Fig. 4A and B demonstrate the reproducibility of results for a particular sample even when the injections are not consecutive. Fig. 4C shows the enhancement of the detection capabilities with a longer injection time. Anodic peaks were also observed for PEG–chymotrypsin. Both protease and chymotrypsin have relatively few (seven and six, respectively) arginine and L-histidine groups resulting in a relatively small residual positive charge. Anodic peaks were not observed for the etched cholesterol capillary. This can be explained by the fact that the measured anodic current for the etched cholesterol capillary [26] is considerably smaller than for the C_{18} column so the protease species observed in Fig. 4 were not detected during a 30 min measurement period at −20 kV.

A further comparison between the data presented in Fig. 1 is shown in Fig. 5 on the etched C_{18} modified capillary for: (A) and (B) PEG–catalase and (C) and (D) PEG–chymotrypsin. The cathodic electrochromatograms (Fig. 5A and C) show a different migration pattern on the C_{18} column from what is obtained for the same PEG proteins on the cholesterol column. This data further substantiates the chromatographic effect of the bonded moiety on the capillary wall in this type of OT-CEC experiment.

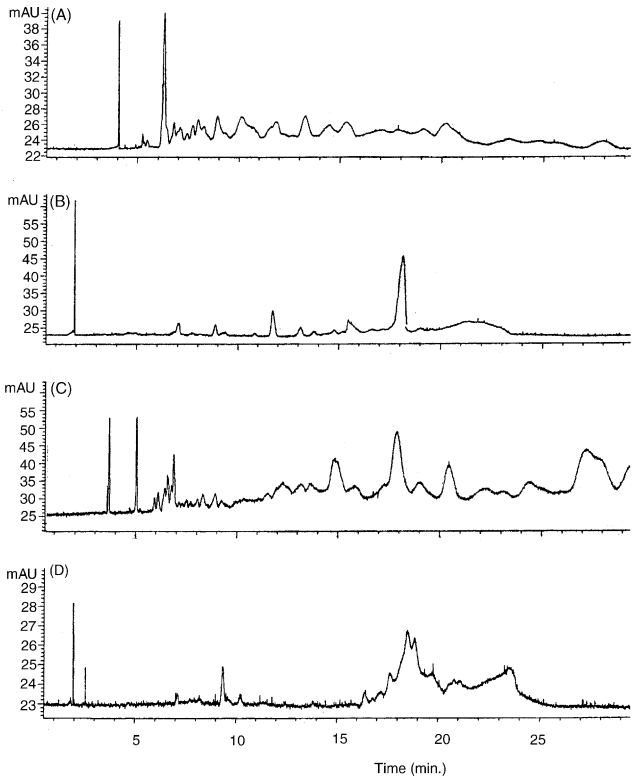

Fig. 5. Electrochromatograms of PEG–catalase on an etched C_{18} modified capillary in pH 2.14 at: (A) 20 kV and (B) −20 kV; and of PEG–chrymotrypsin at: (C) 20 kV and (D) −20 kV.

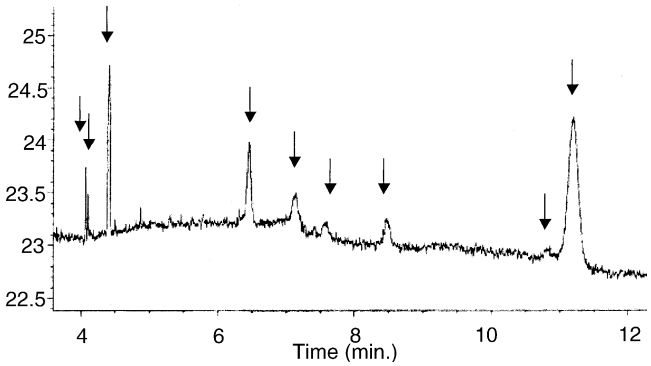

Fig. 6. Electrochromatogram of PEG–superoxide dismutase on an etched C_{18} modified capillary at 20 kV and pH 2.14.

Both the number of species resolved and the apparent elution order are different for the two organic moieties bonded to the etched surface. For each protein, extensively PEGylated species are also obtained so that net migration is in the anodic direction. The data in Fig. 5B and D show the electrochromatograms obtained at −20 kV for the two PEG–proteins.

A final example is the case of superoxide dismutase which has been previously investigated by a combination of capillary electrophoresis and MS [15]. The OTCEC analysis of a PEG–SOD sample on the etched C_{18} capillary is shown in Fig. 6. It appears that at least nine separate species can be identified in the electrochromatogram. This result is an improvement over the earlier study [15] on this same compound which identified eight components. However, it is not known whether the two PEGylated SOD samples were prepared in an identical manner.

A number of variables were tested in arriving at the experimental conditions used to obtain the electrochromatograms shown above. It was generally found that an applied voltage of 20 kV gave the best compromise with respect to resolution and speed of analysis. A number of buffer pH values were also tested from 2.14 to 8.14. For the PEGylated proteins, a pH of 2.14 consistently gave the best results in both the cathodic and anodic modes. Addition of methanol to the running buffer only increased the migration time without any improvement in resolution. These variables have all been shown to be significant factors in optimizing separations in other investigations [22–25]. A summary of the data for each of the PEG–proteins investigated with respect to the number of peaks, the column used, the applied voltage and the buffer pH for the optimum separation achieved are given in Table 2.

Previous studies [22–27] have shown that etched chemically modified capillaries have long column lifetimes (>300 injections) and give reproducible results (R.S.D. < 1.5%) when comparing the migration times of the same solutes for early injections ($n < 10$) to those obtained after considerable use ($200 < n < 300$). Comparable column lifetimes for both the C_{18} ($n \sim 400$) and cholesterol ($n \sim 350$) capillaries

Table 2
List of optimum conditions for each PEGylated protein

PEGylated protein	No. of peaks	Capillary	Voltage (kV)	pH
Chymotrypsin	15	Cholesterol	20	2.14
Protease	25	C_{18}	20	2.14
	28	Cholesterol		
Catalase	25	C_{18}	20	2.14
	27	Cholesterol		
Lipase	7	Cholesterol	30	4.41
Superoxide dismutase	9	C_{18}	20	2.14
Asparaginase	9	Cholesterol	30	2.14

were obtained in this study and the electrochromatograms shown in Figs. 1–6 were reproducible over several consecutive injections as well as with intervening analyses of other samples.

4. Concluding remarks

It has been demonstrated that open tubular capillary electrochromatography is a viable technique for the analysis of PEGylated proteins. It is a rapid method (<30 min) that provides high resolution for these potentially complex mixtures. The process is reproducible and the columns are rugged. This technique could easily be used for verifying the purity of PEG–proteins used as therapeutic agents or it could establish batch-to-batch reproducibility of different lots of the drug.

Acknowledgements

The financial support of the National Institutes of Health (NIH MBRS Grant 0819210-21 and Grant GM 0063519-01) and the National Science Foundation (CHE 0094983) is gratefully acknowledged. J.J.P. would like to acknowledge the support of the Camille and Henry Dreyfus Foundation through a Scholar Award.

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