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High-performance capillary electrophoresis of proteins

Sodium dodecyl sulphate–polyacrylamide gel-filled capillary column for the determination of recombinant biotechnology-derived proteins

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ABSTRACT

Fused-silica capillary columns were filled with sodium dodecyl sulfate–polyacrylamide gel and the column effluent was monitored at 214 nm using a commercially available high-performance capillary electrophoresis (HPCE) instrument to separate and rapidly quantify recombinant biotechnology-derived proteins. An excellent linear relationship ($r > 0.999$) exists between the peak migration time and the molecular weights of reference proteins in the range 10 000–100 000 and 40 000–200 000 dalton by use of the capillary columns filled with acrylamide gel at a T composition of 5% and 3%, respectively. The relative standard deviation (R.S.D.) of the peak migration time is *ca.* 1%. Theoretical plates of $5 \cdot 10^5$–$1 \cdot 10^6$ per metre are routinely being obtained. Calibration graphs of peak area *versus* weight of recombinant biotechnology-derived proteins are linear ($r > 0.999$) and the proteins may be quantified with an R.S.D. of *ca.* 3–7%. As little as 50 nmol of a protein may be quantified and an impurity peak of molecular weight *ca.* 1500 less than that of the parent compound (*ca.* 60 000 dalton) may be differentiated by HPCE with a gel-filled capillary column.

INTRODUCTION

Biotechnology-derived proteins are posing a considerable challenge to analytical chemistry as the separation efficiencies of high-performance liquid chromatography (HPLC) for these proteins are far less than those of lower molecular weight compounds [1]. Electrophoresis, especially polyacrylamide gel (PAG) electrophoresis, is an indispensable technique for the separation of proteins based on their apparent molecular weights. In spite of impressive advances made in recent years [1], electrophoresis still represents a collection of labor-intensive and time consuming techniques and is not readily automatable. Casting of a gel, application of samples, electrophoresis and staining of gels are time-consuming tasks prone to irreproducibility and quantification by means of an optical scanning apparatus is often sub-optimum.

Recent developments in the commercialization of high-performance capillary

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electrophoresis (HPCE) instruments for capillary zone electrophoresis (CZE) [2–5] have made it possible to exploit potential of sodium dodecyl sulfate (SDS)-PAG-filled capillary columns for the analysis of recombinant biotechnology-derived protein with promises of rapid and automated analysis of multiple samples and improved reproducibility and quantification. Although several U.S. patents have been issued for the preparation of gel-filled capillary columns [6–8], mainly for nucleotide analysis [9–12], no such column has been made commercially available. Cohen and Karger [13] applied gel-filled capillaries to the electrophoresis of peptides and proteins, but no quantitative data were presented.

This paper describes the preparation of SDS-PAG-filled capillary columns and the application of such columns to the electrophoretic separation and quantification of recombinant biotechnology-derived proteins using a commercially available HPCE instrument.

EXPERIMENTAL

HPCE instrumentation

A P/ACE System 2000 HPCE instrument (Beckman, Palo Alto, CA, U.S.A.) was used to obtain analytical data. A Model 270A Capillary Electrophoresis System (Applied Biosystems, Foster City, CA, U.S.A.) was used to develop and evaluate the SDS-PAG composition for the longevity and performance of gel-filled capillary columns (GFC).

Each HPCE-GFC run involves a 10-s electrokinetic injection (−2.5 kV) of a protein sample into a GFC and monitoring compounds migrating in the column effluent at 214 nm. The temperature in a column cartridge was maintained at 25°C and an electrophoretic run was conducted at −2.0 to −3.0 kV (−83 to −130 V/cm, 12 μA) with a running buffer. The area under the protein peak was integrated by means of a program in a VAX mainframe computer.

Reagents

Phosphate-buffered saline (PBS), sample solubilization buffer (SSB) and running buffer solution were made from stock reagent solutions [1]. An acrylamide stock solution containing 30% acrylamide and 0.8% bisacrylamide was obtained from Enprotech (Hyde Park, MA, U.S.A.).

Acrylamide buffer solution [1,14] contained either 5.1% T and 2.6% C or 3.1% T and 2.6% C with 375 m*M* Tris (pH 8.8), 3.2 m*M* SDS and 2.35 *M* ethylene glycol. The percentage of T and C in the acrylamide gel solution was calculated using the equations

$$\text{T (\%)} = \text{acrylamide (g)} + \text{bisacrylamide (g) per 100 ml of solvent}$$

$$\text{C (\%)} = \frac{\text{bisacrylamide (g)} \cdot 100}{\text{bisacrylamide (g)} + \text{acrylamide (g)}}$$

The presence of ethylene glycol in the gel formulation as well as in the running buffer minimizes the formation of bubbles during electrophoresis and thus significantly improves the longevity of the GFC. The acrylamide buffer solution was

deaerated by sparging with dry helium for 60 s. Appropriate amounts of ammonium peroxodisulfate solution (Bio-Rad Labs., Richmond, CA, U.S.A.) and N,N,N′,N′-tetramethylethylenediamine (TEMED) solution (Bio-Rad Labs.) were then added to the acrylamide buffer solution to initiate slow polymerization.

PBS contains 150 m*M* NaCl, 2.8 m*M* NaH_2PO_4 and 7.8 m*M* Na_2HPO_4 and 4X SSB consists of 62.5 m*M* Tris (pH 6.8) and 12.8 m*M* SDS. The running buffer contains 300 m*M* Tris (pH 8.8), 3.2 m*M* SDS and ethylene glycol. Just prior to an electrophoretic run, the running buffer is deaerated by sonication under vacuum.

Preparation of gel-filled capillaries (GFC)

A roll of fused-silica capillary tubing (50 μm I.D., 375 μm O.D.) was obtained from Polymicro Technologies (Phoenix, AZ, U.S.A.). Capillaries were cut to a few centimeters longer than the final column length of 24 cm and a detector window was created at *ca.* 2 cm longer than the effective column length of 7 cm by removing the polyimide coating with a polyimide stripper (Model S200, Polymicro).

The inner surface of the capillaries was first treated with 2 *M* H_2SO_4 by heating at 200°C for 15 min to prepare the surface for later attachment of bifunctional groups. A bifunctional reagent, γ-(acryloxypropyl)methyldichlorosilane (Petrarch Systems, Bristol, PA, U.S.A.), 9 m*M* in toluene, was attached to the capillaries by reaction at 100°C for 10 min. The bifunctional reagent silanizes the inner glass surface to minimize adsorption of protein and covalently bonds the polyacrylamide gel to the capillary wall [15,16]. Polyethylene tubing (0.38 mm I.D., 1.09 mm O.D.) (Clay Adams, Parsippany, NJ, U.S.A.) was connected to the capillary and a micro-syringe pump was used (Hamilton, Reno, NV, U.S.A.) to facilitate column rinsing and filling.

After the capillary had been treated with the bifunctional reagent, new polyethylene tubing was attached to both ends of the capillary. Just prior to the initiation of the polymerization of the gel, the columns were rinsed and the inner, silanized capillary surfaces wetted with dry methanol. The column was then filled with water. Wetting the inner, silanized surface is an essential operation in order to consistently obtain air-bubble-free, well packed columns.

Just prior to each rinsing and filling operation, *ca.* 0.5 cm of the polyethylene tubing was cut from the inlet side to minimize the introduction and trapping of air bubbles in the capillary. Immediately after the capillary had been filled with the acrylamide gel solution, the polyethylene tubings attached to both ends of the columns were cramped tightly with hemostatic forceps (VWR Scientific, Chicago, IL, U.S.A.). The gel-filled capillary columns thus prepared were left to stand vertically at room temperature for 48–72 h. The idea is to cure the column and to allow fluid, induced by shrinkage of the gel, to migrate to an upper end of the column. The top end of the column is then cleared to remove the fluid. The gel-filled capillary column was installed in a capillary column cartridge (Beckman) and stored by immersion of both ends of the capillary in the running buffer. Just prior to an electrophoretic run, both ends of the capillary column were cut squarely so as to have lengths of 7.0 cm (inlet) and 17 cm (outlet) from the detection window. The column was conditioned at −50 V/cm for 60 min.

Separation of proteins

Both the high- and low-molecular-weight reference protein standard solutions

(Bio-Rad Labs.) used contained a mixture of proteins: hen egg white lysozyme (molecular weight 14 400 dalton), soybean trypsin inhibitor (21 500), bovine carbonic anhydrase (31 000), hen egg white ovalbumin (45 000), bovine serum albumin (BSA) (66 200), rabbit muscle phosphorylase *b* (97 400), *Escherichia coli* β-galactosidase (116 250) and myosin (200 000). Samples of recombinant biotechnology-derived protein used were manufactured by Upjohn (Kalamazoo, MI, U.S.A.). They are (1) soluble CD_4 (molecular weight 20 395 dalton), which contains 183 amino acids, and (2) soluble CD_4-PE (molecular weight 59 187 dalton), which consists of 545 amino acids and contains the domains I and II of the CD_4 molecule [17] and the domains II and III of the *Pseudomonas* exotoxin A [18]. Protein samples were diluted in PBS to an appropriate concentration and made up to a final volume of 20 μl with 4X SSB. The protein samples were heated at 55 ± 5°C for 10 min to denature them.

RESULTS AND DISCUSSION

Longevity of gel-filled capillary columns

Without the additive, ethylene glycol, an SDS-PAG-filled capillary column forms bubbles within a few minutes and the flow of electric current ceases. The presence of ethylene glycol in SDS-PAG at a level of 1.8–2.7 *M* significantly improves the longevity of the GFC. For example, one column lasted over 2 weeks of continuous electrophoresis and another quantified over 300 protein samples. The column performance gradually deteriorates, however, after *ca.* 40 sample analyses. Below a 1.8 *M* ethylene glycol concentration, the lifetime of a GFC is short and ethylene

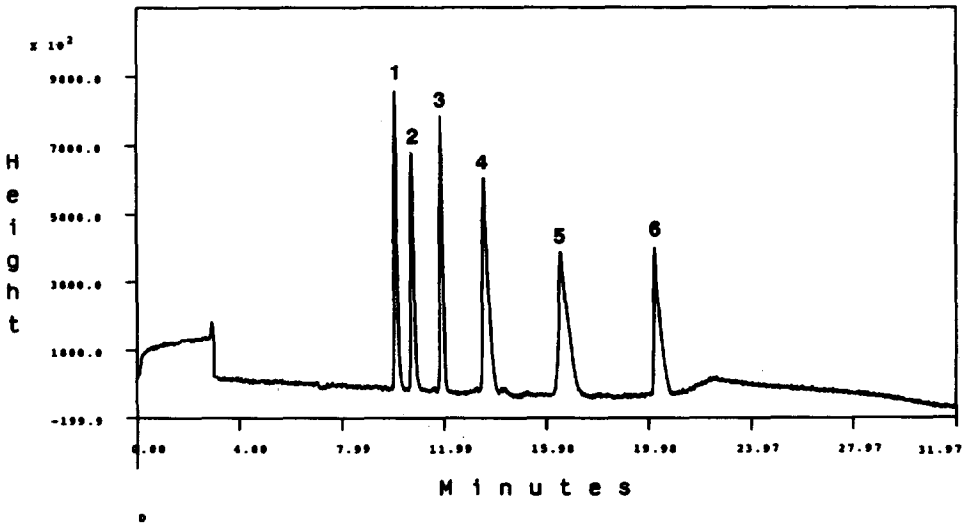

Fig. 1. Separation of molecular weight reference standards by HPCE-GFC as monitored at 214 nm. Conditions: −83 V/cm; 12 μA; column temperature, 25°C; migration distance, 7 cm; fused-silica capillary, 75 μm I.D.; running buffer, 375 m*M* Tris (pH 8.8)–0.1% SDS–ethylene glycol. Molecular weight standards used: 1 = lysozyme (mol. wt. 14 400 dalton); 2 = trypsin inhibitor (21 500); 3 = carbonic anhydrase (31 000); 4 = ovalbumin (45 000); 5 = BSA (66 200); 6 = phosphorylase *b* (97 400).

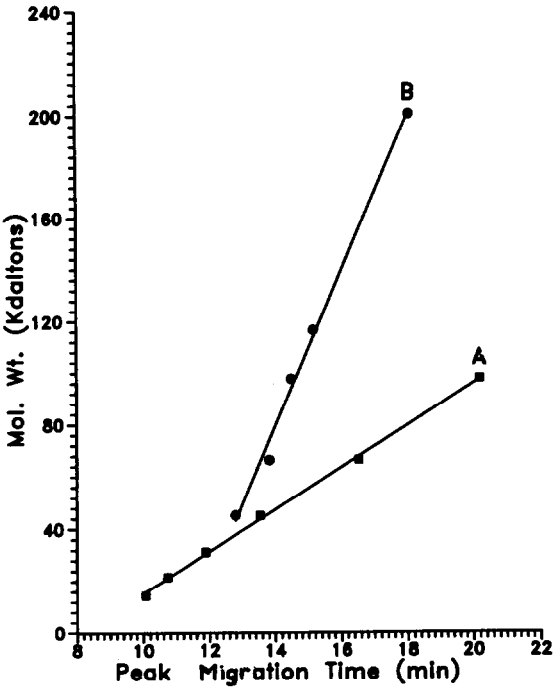

Fig. 2. Molecular weight calibration graphs (line A, 5% T; line B, 3% T) for HPCE-GFC indicating the existence of a linear relationship ($r > 0.999$) between peak migration time and molecular weight. Molecular weight standards used: lysozyme, trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase *b*, β-galactosidase and myosin. Conditions: −83 V/cm; 12 μA; column temperature, 25°C; migration distance, 7 cm; fused-silica capillary, 75 μm I.D.; running buffer, 375 m*M* Tris (pH 8.8)–0.1% SDS–ethylene glycol.

glycol concentrations over 2.7 *M* results in a very soft gel with a significant loss of peak resolution. The running buffer may contain over 4 *M* ethylene glycol with no loss of theoretical plates. However, the SDS-PAG composition developed for capillary electrophoresis is not the ultimate one. The lifetime of a column is still not predictable. Frequently, a tiny bubble forms at the detector window, resulting in significant deterioration of the column performance even though no loss of current flowing through the column can be detected. During a prolonged HPCE-GFC run, the gel becomes extremely soft and fluid due to Joule heating and soft/fluid gel tends to drip out of the capillary column. Fluidity is especially pronounced with the gel of 3% T composition.

Wetting of the inner surface of the silanized capillary column with methanol has been a key factor contributing to dependable and consistent preparation of GFCs.

Molecular weight reference standards

Capillary columns filled with SDS-PAG of 5% and 3% T composition may be used to determine the apparent molecular weights of proteins, ranging from 10 000 to 100 000 and 40 000 to 200 000 dalton, respectively. A typical HPCE-GFC electropherogram indicating the separation of molecular weight reference protein standards

TABLE I

PRECISION OF THE PEAK MIGRATION TIME FOR MOLECULAR WEIGHT PROTEIN STANDARDS

Run No.	Peak migration time (min) for proteins A–F[a]					
	A	B	C	D	E	F
1	14.82	15.68	17.28	20.00	25.81	29.78
2	14.44	15.75	17.42	20.18	25.48	30.18
3	14.52	15.88	17.55	20.41	25.90	30.73
4	14.65	16.00	17.63	20.59	26.12	31.06
5	14.41	15.74	17.41	20.22	25.60	30.30
6	14.23	15.55	17.20	20.02	25.38	30.84
R.S.D. (%)	1.4	0.99	0.92	1.1	1.1	1.6

[a] Proteins: A = lysozyme (mol. wt. 14 400 dalton); B = trypsin inhibitor (21 500); C = carbonic anhydrase (31 000); D = ovalbumin (45 000); E = BSA (66 200); F = phosphorylase *b* (97 400).

using an SDS-PAG (5% T) filled capillary is shown in Fig. 1. The existence of a linear relationship ($r > 0.999$) was noted when the peak migration time was plotted against the molecular weights of the protein standards (Fig. 2).

The precision of the protein peak migration time was determined by repeated injection of the protein reference molecular weight standard solution containing lysozyme, trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA and phosphorylase b. The relative standard deviation of the peak migration time ranged from 0.92 to 1.6% (Table I).

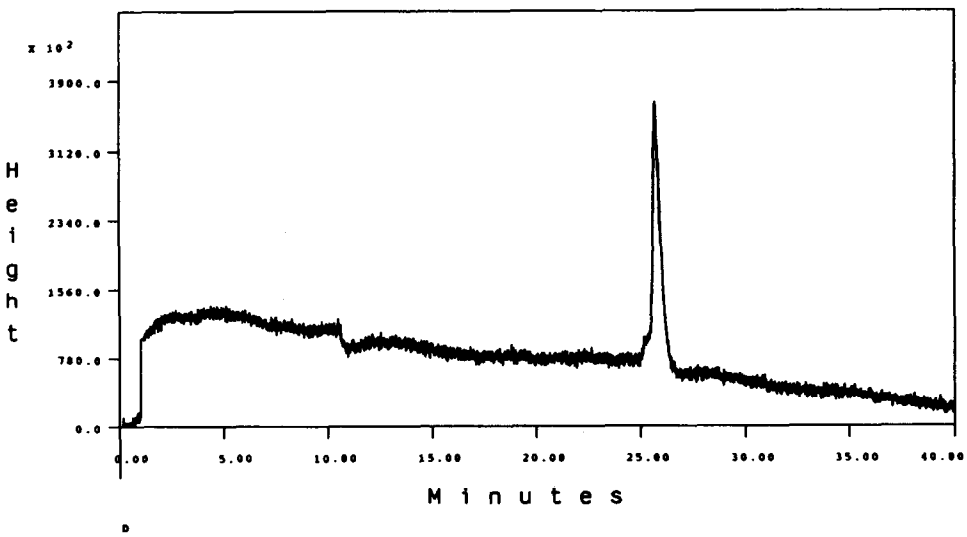

Fig. 3. HPCE-GFC separation of a recombinant biotechnology-derived protein (soluble CD_4-PE) as monitored at 214 nm. Conditions: −83 V/cm; 12 μA; column temperature, 25°C; migration distance, 7 cm; fused-silica capillary, 75 μm I.D.; running buffer, 375 m*M* Tris (pH 8.8)–0.1% SDS–ethylene glycol.

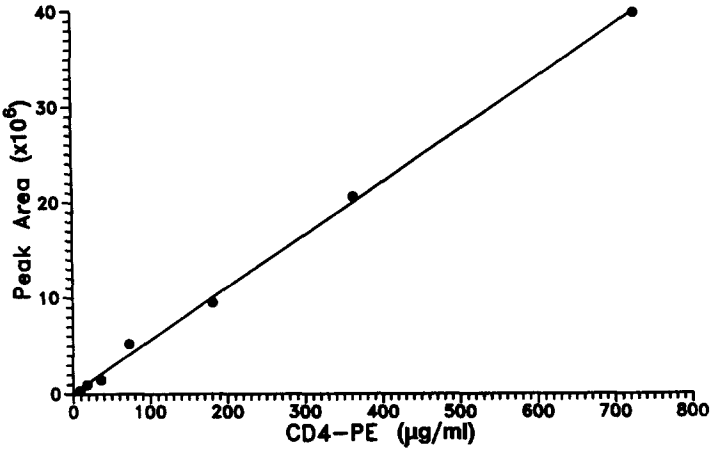

Fig. 4. Calibration curve for the determination of soluble CD_4-PE, indicating the existence of a linear relationship ($r > 0.999$) between the weight of sCD_4-PE and the peak area as monitored at 214 nm. Conditions: −83 V/cm; 12 µA; column temperature, 25°C; migration distance, 7 cm; fused-silica capillary, 75 µm I.D.; running buffer, 375 m*M* Tris (pH 8.8)–0.1% SDS.

Determination of recombinant biotechnology-derived proteins

A typical electropherogram of a recombinant biotechnology-derived protein, soluble CD_4-PE is shown in Fig. 3. HPCE-GFC detected the presence of a dimer in a sample of sCD_4 (data not shown) and demonstrated the capability of detecting an impurity peak with a molecular weight *ca.* 1500 less than that of the parent compound of *ca.* 60 000 dalton in a sample of sCD_4-PE (Fig. 3).

The ability of HPCE-GFC to quantify proteins was examined. The calibration curve for sCD_4-PE is linear ($r > 0.999$) in the range 10–725 µg/ml protein (Fig. 4). The line intersected the *x*-axis at near zero, indicating minimum adsorption of the protein on the column wall. The calibration curve for sCD_4 is also linear ($r > 0.999$) between 20 and 2000 µg/ml protein (data not shown). A linear assay range of two orders of magnitude of protein concentration has thus been demonstrated. The detector became saturated at sCD_4 concentrations above 3 mg/ml. As little as 50 nmol of pro-

TABLE II

PRECISION OF HPCE-GFC FOR THE ASSAY OF sCD_4-PE

Run No.	Peak migration time (min)	Peak area
1	15.77	7 220 000
2	15.87	7 761 000
3	15.93	7 172 000
4	15.97	7 405 000
5	16.02	7 578 000
6	16.08	7 774 000
7	16.07	7 196 000
R.S.D. (%)	0.70	3.53

teins may be quantified by HPCE-GFC. This relatively high sensitivity of HPCE-GFC to protein samples was obtained by monitoring the migration of the proteins at 214 nm.

The precision for the assay of a biotechnology-derived protein, sCD_4-PE, was evaluated by repeated injection of samples. The relative standard deviation (R.S.D.) of the peak migration time was less than 1% and that for the assay of the protein is *ca.* 3.5% (Table II). The precision for the HPCE-GFC assay of CD_4 is similar (data not shown). In order to improve the precision of the assay, an increase in the electrokinetic injection time and/or protein concentration was attempted. However, the R.S.D. of the assays stubbornly remained between 3 and 7%. Inclusion of an internal standard may be needed to further improve the precision of the assay. As the instrument has no provision for cooling the samples, an increase in temperature of the protein sample solution was observed during a long HPCE-GFC run, such as an overnight run. The questionable stability of proteins and the potential for adsorption of protein onto the plastic sample cup (Beckman) under such adverse HPCE-GFC running conditions may be a factor to be recognized for development of the assay method. Efforts are being made to improve the precision of the HPCE-GFC assay of proteins.

CONCLUSION

HPCE-GFC has been demonstrated to be capable of separating and determining the apparent molecular weights of impurities and of quantifying recombinant biotechnology-derived proteins in a relatively short time. Hence HPCE-GFC may become an indispensable tool for monitoring protein purification processes and for determining the stability characteristics of a pharmaceutical product containing a recombinant biotechnology-derived protein.

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