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Sodium dodecyl sulfate polyacrylamide gel- and replaceable polymer-filled capillary electrophoresis for molecular mass determination of proteins of pharmaceutical interest

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Abstract

Performance of commercially available replaceable SDS polymer-filled capillary electrophoresis (rPGCE) systems were evaluated for the determination of the molecular mass of proteins of pharmaceutical interest. The mass values obtained by these systems were compared with those obtained by SDS-PAGE and high-performance size exclusion chromatographic (HPSEC). The molecular masses of proteins determined by rPGCE were not significantly different and agreed closely with those obtained by SDS-PAGE and with the theoretical values of the proteins. HPSEC gave the least reliable data. Performance of the SDS crosslinked polyacrylamide gel-filled capillary electrophoresis system was superior to that of the commercially available rPGCE systems. Nevertheless, the rPGCE systems present significant advantages over the conventional SDS-PAGE and HPSEC methods for rapid analysis and improved precision for the determination of the molecular masses of proteins of pharmaceutical interest.

1. Introduction

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) is an indispensable technique for separation of proteins based on their apparent molecular mass [1]. However, SDS-PAGE includes a number of labor-intensive and time consuming techniques and quantification by means of an optical scanner is often sub-optimal. High-performance size-exclusion chromatography (HPSEC) is routinely employed to determine the composition of proteins. However, peak resolution performance of HPSEC is not always ideal [2].

Advances in high-performance capillary electrophoresis (HPCE) instrumentation [3–6] have enabled the use of SDS polyacrylamide gel- and/or replaceable polymer-filled capillary electrophoresis systems for the separation of proteins. The application of these techniques should provide rapid and automated analysis with improved reproducibility and quantification. Hjertén was the first to utilize a polyacrylamide gel-filled capillary column to demonstrate separation of a membrane protein [7]. Cohen and Karger [8] applied SDS polyacrylamide gel-filled capillaries for electrophoresis of peptides and proteins. Neither publications presented quantitative data. Tsuji prepared SDS crosslinked polyacrylamide gel-filled capillary columns and provided data for molecular mass separation and quantitation of recombinant proteins [9].

Zhu *et al.* [10] exploited the molecular sieving action of non-acrylamide polymers, *e.g.* dextran,

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methylcellulose, and polyethylene glycol [11], and used them as additives in the HPCE buffer to facilitate separation of DNA and proteins. Ganzler *et al.* also examined a branched dextran and linear polyethylene glycol polymer networks and successfully achieved separation of proteins [12]. Bode demonstrated the sieving effect of a linear polyacrylamide for separation of RNA and proteins based on their molecular masses [13,14]. Widhalm *et al.* used a non-derivatized fused-silica capillary column and applied a linear polyacrylamide for electrophoresis of proteins [15]. Since no attempt was made to eliminate the electroosmotic flow, data presented were at best preliminary. Just recently such a linear polyacrylamide gel- and/or replaceable polymer-filled capillary systems has become commercially available [16,17]. This paper examines the performance of SDS crosslinked polyacrylamide gel- and replaceable polymer-filled capillary electrophoresis systems and explores their applicability for the molecular mass determination of proteins of pharmaceutical interest.

2. Experimental

2.1. Reagents

The molecular mass protein standard solution containing *ca.* 200 μg each of hen egg white lysozyme (molecular mass: 14 400 Da), soybean trypsin inhibitor (21 500), bovine carbonic anhydrase (31 000), hen egg white ovalbumin (45 000), bovine serum albumin (BSA) (66 200), and rabbit muscle phosphorylase b (97 400) per 100 μl was obtained from Bio-Rad (catalogue no. 161-0304, SDS-PAGE low range molecular weight standard).

A 5-μl aliquot of the molecular mass protein standard solution (Bio-Rad) was diluted in 40 μl of the sample buffer solution containing 1% SDS in 0.12 *M* Tris-HCl, pH 6.6. After thorough mixing, 2 μl of 2-mercaptoethanol (Sigma) was added for reduction and the mixture was denatured by heating at 80°C for 5 min. The concentration of each of the six proteins in the diluted, reduced, and denatured solution was approximately 0.2 μg/μl.

2.2. Preparation of protein samples

Samples of rbSt (recombinant bovine somatotropin, molecular mass: 21 812), sCD_4-PE (molecular mass: 59 172), and ATGAM (polyclonal anti-thymocyte equine immune globulin, molecular mass: *ca.* 150 000), used in this study were all manufactured by The Upjohn Company (Kalamazoo, MI, USA). The sCD_4-PE is a chimeric protein and consists of 545 amino acids and contains the domains I and II of the CD_4 molecule [18] and the domains II and III of the *Pseudomonas* exotoxin A [19].

Protein samples were diluted in the sample buffer solution to a concentration of approximately 0.1–0.5 μg/μl. To reduce and denature the protein, 2 μl of 2-mercaptoethanol was pipetted into a 50-μl volume of the protein solution. After thorough mixing, the sample was heated at 80°C for 5 min. For preparation of a rbSt sample, approximately 1 mg of rbSt was dissolved in 100 μl of a weak ammonium solution [10 μl of NH_4OH (Mallinckrodt) per 10 ml of water (Burdick and Jackson, Muskegon, MI, USA)]. This rbSt sample solution was further diluted in sample buffer solution to a concentration of *ca.* 0.5 μg protein/μl, then reduced and denatured just prior to analysis.

2.3. Instrumentation

A Beckman P/ACE system 2100 high-performance capillary electrophoresis (HPCE) instrument (Beckman Instruments, Fullerton, CA, USA) was used.

SDS crosslinked polyacrylamide gel-filled capillary system

A coated column for the SDS crosslinked polyacrylamide gel electrophoresis was prepared as described by Tsuji [9]. The acryloxypropylsilane reacted column (27 cm × 75 μm I.D. × 375 μm O.D.; effective length 7 cm) was filled with an SDS acrylamide solution (5% T, 2% C) containing ethylene glycol (Aldrich, Milwaukee, WI, USA). Samples were electrokinetically injected onto the capillary column for 10 s at −2.5 kV and proteins migrating in the column

were monitored at 214 nm. Column temperature was maintained at 25°C by a circulating coolant and electrophoresis was conducted at −75 V/cm (12 μA) using a running buffer composed of 100 m*M* Tris (pH 8.8), 0.1% SDS, and 2.5 *M* ethylene glycol as reservoirs at both the anode and the cathode ends.

Beckman replaceable SDS polymer-filled capillary system

Each analytical run consists of rinsing a coated capillary column (Cat. No. 241521, Beckman; 47 cm × 100 μm I.D. × 375 μm O.D.; effective length, 40 cm) with water for 2 min followed by a 2-min wash with 1.0 *M* HCl. The column was then filled with the SDS polymer solution (eCAP SDS 200, Cat. No. 241522 or eCAP SDS 14-200, Beckman) [16] for 4 min. Use of a coated capillary column is required to eliminate electroosmosis.

Column temperature was maintained at 20°C by a circulating coolant to minimize band diffusion and ensure effective size separation. An electrophoretic run was conducted at −300 V/cm (24 μA) using the SDS polymer solution (Beckman) as reservoirs at both the anode and the cathode ends. The molecular mass protein standard and sample solutions were injected for 60 s under nitrogen pressure (total injection volume, *ca.* 40–50 nl) onto the SDS polymer-filled capillary column.

ABI replaceable SDS polymer-filled capillary system

A bare fused-silica capillary column (27 cm × 50 μm I.D. × 375 μm O.D.; effective length, 20 cm, Polymicro Technologies, Phoenix, AZ, USA) was activated by a 0.1 *M* NaOH solution for 15 min. The analytical runs were started by coating the capillary with the polymer solution (Cat. No. 401482, Applied Biosystems, ABI, Foster City, CA, USA) for 60 min. Since the polymer formulation contains a linear acrylamide polymer [17] to minimize the electroosmotic flow, use of a specially coated capillary column is not required. After a couple of electrophoretic runs, the capillary was rinsed with water and regenerated with 1 *M* HCl followed by 0.1 *M* NaOH for 15 min each.

Column temperature was maintained at 30°C by a circulating coolant. Each electrophoretic run was conducted at −300 V/cm (37 μA) using the polymer solution (ABI) as reservoirs at both the anode and the cathode ends. The molecular mass protein standard and sample solutions were injected for 60 s under nitrogen pressure (total injection volume, *ca.* 10 nl) onto the SDS polymer-filled capillary column.

Peaks migrating in the capillary columns were monitored on-column by UV at 214 nm. The area under the peak was integrated by means of an in-house GC/LC program residing on a VAX mainframe computer and with an electronic integrator (Model 3392A, Hewlett-Packard, Palo Alto, CA, USA).

SDS-PAGE

For the SDS-PAGE analysis, procedures described in the Current Protocols in Molecular Biology [1] were followed. After the silver staining, each slab-gel plate was scanned with a densitometer (Model 300b, Hewlett-Packard) and the molecular mass values of the proteins were calculated using ImageQuant software (Molecular Dynamics, Sunny Vale, CA, USA).

HPSEC

A Series II 1090 liquid chromatograph equipped with a low-volume autosampler–injector (Hewlett-Packard) was used for the high-performance size-exclusion chromatographic (HPSEC) assay. A Zorbax GF-250 column (25 cm × 9.4 mm I.D.; MAC-MOD Analytical, Chadds Ford, PA, USA) was used for the analysis of rbSt and sCD_4-PE; a Bio-Sil TSK-250 column (60 cm × 7.5 mm I.D., Cat. No. 125-006, Bio-Rad Laboratories, Richmond, CA, USA) was used for ATGAM.

A thoroughly de-gassed mobile phase composed of 150 m*M* NaCl (Mallinckrodt, Paris, KY, USA), 25 m*M* NaH_2PO_4 (J.T. Baker Chemical Co., Phillipsburg, NJ, USA) at pH 8.0 and 0.1% SDS was pumped at a flow-rate of approximately 1.0 ml/min. Protein solutions were prepared in 0.1% SDS at a concentration

of ca. 10 mg/ml. A 100-μl aliquot of the sample solution was injected onto the size-exclusion column. Peaks of ATGAM and rbSt eluting from the column were monitored at 280 nm and sCD_4-PE was monitored at 221 nm.

3. Results and discussion

In the presence of SDS proteins form SDS–protein complexes with a constant SDS/protein ration of 1.4 g/g [1]. Thus, separation of SDS–protein complexes through the gel or polymer matrix is strictly based on their size [1,13,14].

3.1. Comparison of SDS crosslinked polyacrylamide gel- and polymer-filled capillary electrophoresis systems

The following SDS crosslinked polyacrylamide gel- and replaceable SDS polymer-filled capillary electrophoresis systems were evaluated: (A) a SDS polyacrylamide gel [9], (B) 2 polymers from Beckman [16], (i) eCAP SDS 200 and (ii) eCAP SDS 14-200, and (C) one from ABI [17], containing a linear acrylamide polymer. The SDS crosslinked polyacrylamide gel-filled capillary system utilizes a 27 cm × 75 μm I.D. (acryloxy silane coated) column [9]. Both Beckman systems use a proprietary coated 47 cm × 100 μm I.D. column [16], and the ABI system employs a 27 cm × 50 μm I.D. bare fused-silica capillary column. The crosslinked polyacrylamide gel system was operated at a column temperature of 25°C, the Beckman systems at 20°C, and the ABI system at 30°C, respectively. Since different optimum temperature profiles were demonstrated for the maximum performance of the polymer-filled capillaries [20], the procedure published [9] and the protocols/instructions provided by the manufacturers were assumed to represent the optimum conditions and were used without further modification.

Typical electropherograms indicating separation of the six molecular mass protein standards by the SDS crosslinked polyacrylamide gel- and by two commercially available replaceable polymer-filled capillary systems are presented in Figs. 1-I, 1-IIA, 1-IIB, and 1-III. Although the column was the shortest, *i.e.* 7 cm, peak migration time of the proteins through the SDS crosslinked polyacrylamide gel-filled column was the longest (Fig. 1-I). The slow migration of the proteins may be attributed to the low voltage, essential to minimize bubble formation to extend the life time of the column [9]. The shortest analysis time was achieved by the ABI system, which utilizes a higher voltage (*i.e.* 14.1 kV) and a shorter column, *i.e.* 20 cm; all 6 proteins migrated in less than 10 min (Fig. 1-III).

Performance of the SDS gel- and polymer-filled capillary systems was evaluated by calculating the theoretical plate number (N) and peak resolution (R_s) following the methods described in the USP [21]. Since the capillary columns used showed variance with respect to length and inner diameter, N was expressed per voltage (kV) for comparative purposes. Since N can be expressed by the following equation:

$$N = (\mu_{ep}V)/2D \quad \text{(see Ref. 22)}$$

where N is the theoretical plate number, μ_{ep} is the electrophoretic mobility of the solute, V is the voltage, and D is the diffusion coefficient of the solute.

Thus, N is a function of the voltage and is independent of column length. As shown in Table 1, the highest N values for the lysozyme and the carbonic anhydrase peaks were obtained by the SDS crosslinked polyacrylamide gel-filled system followed by the ABI system. The high theoretical plate numbers obtained by these gel- and polymer-filled capillary systems may be due to the limited diffusion of the high-molecular-mass protein complexes through the matrix [23].

A superb R_s value between the lysozyme and the trypsin inhibitor peaks was obtained by all the gel- and the polymer-filled systems evaluated (Table 1). However, base-line resolution between the BSA (66 200 Da) and the phosphorylase b (97 400 Da) peaks was difficult to achieve by the Beckman eCAP SDS 200 system (Fig. 1-IIA). The peak resolution was improved by the eCAP SDS 14-200 system (Fig. 1-IIB). No difficulty in resolving the two peaks was ex-

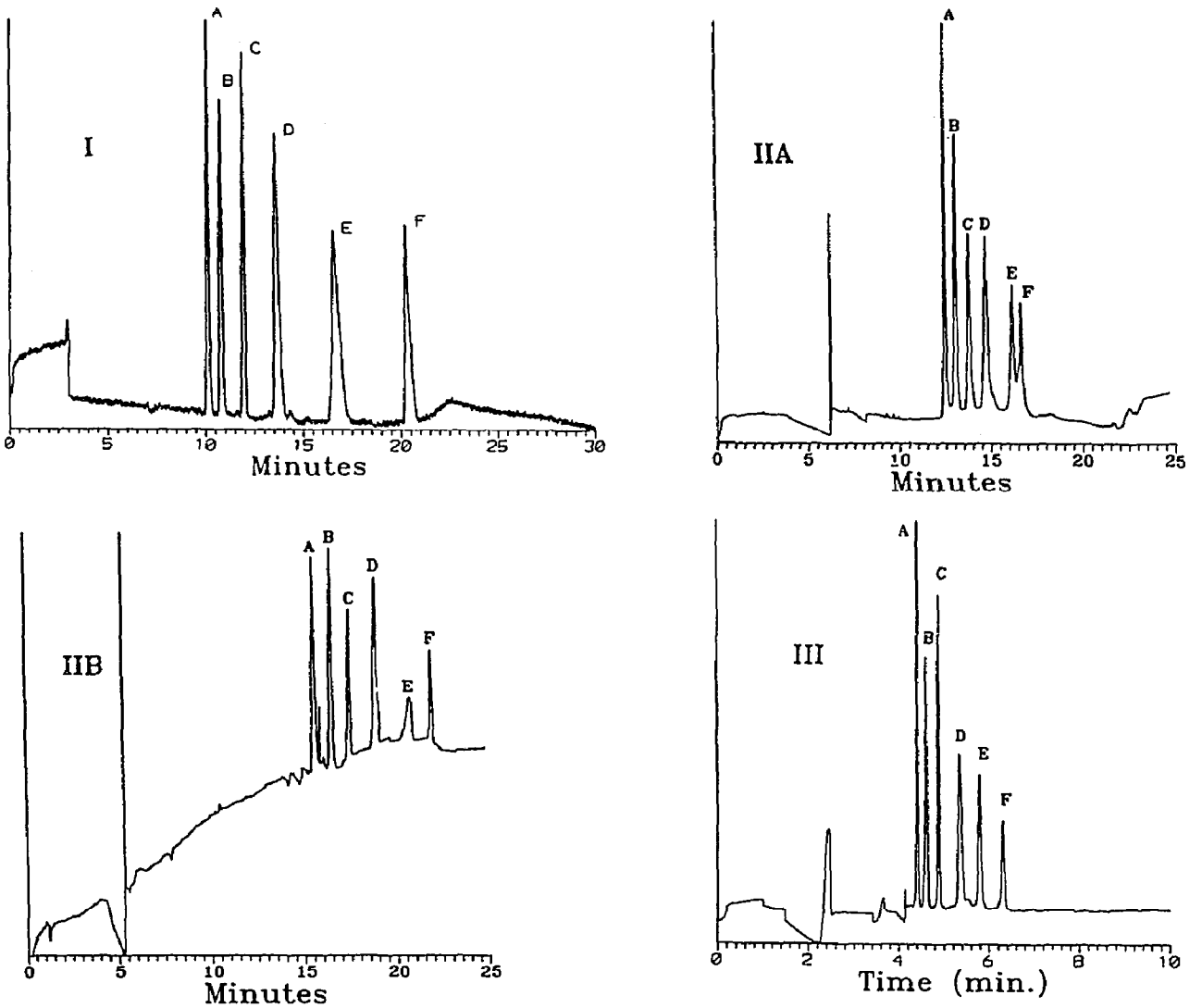

Fig. 1. Typical electropherograms of the SDS polyacrylamide gel- and/or replaceable SDS polymer-filled capillary electrophoresis of the molecular mass protein reference standards as monitored at 214 nm. Peak migration order: A = lysozyme (14 400 Da); B = trypsin inhibitor (21 500); C = carbonic anhydrase (31 000); D = ovalbumin (45 000); E = BSA (66 400); and F = phosphorylase b (97 400). (I) SDS crosslinked polyacrylamide gel-filled capillary column [9]. Conditions, −83 V/cm; column temperature: 25°C; peak migration distance: 7 cm; 75 μm I.D.; inner wall of the capillary was coated with acryloxypropylmethyldichlorosilane–acrylamide and the column was filled with SDS polyacrylamide gel (5% T, 2% C) containing 2.5 *M* ethylene glycol; running buffer: 375 m*M* Tris, pH 8.8, 0.1% SDS, 2.5 *M* ethylene glycol. (II) SDS polymer-filled capillary system from Beckman [(A) SDS eCAP 200, (B) SDS eCAP 14-200] [16]. Conditions, −300 V/cm; column temperature: 20°C; effective peak migration distance: 40 cm; coated capillary: 100 μm I.D.; running buffer: SDS polymer solution from Beckman. (III) SDS polymer-filled capillary system from ABI [17]. Conditions: −300 V/cm; column temperature: 30°C; effective peak migration distance: 20 cm; bare fused-silica capillary: 50 μm I.D.; running buffer: SDS polymer solution from ABI.

perienced by the polyacrylamide gel- and the ABI systems, however.

Complete resolution of 3 minor peaks associated with a sample of carbonic anhydrase was attained by the SDS crosslinked polyacrylamide gel-filled capillary column (Fig. 1-I) but not by the replaceable polymer-filled capillary systems examined (Figs. 1-II, 1-II). Although the crosslinked polyacrylamide gel-filled column gave superb performance, the column suffered from a

Table 1
Theoretical plate number (N) and peak resolution (R_s) of the replaceable SDS polymer-filled capillary electrophoresis systems

Capillary columns	Theoretical plates (N)		Peak resolution (R_s)	
	Lysozyme	Carbonic anhydrase	Lysozyme/trypsin inhibitor	BSA/phosphorylase b
Cross-linked polyacrylamide	50 100	95 900	3.2	7.1
ABI	16 100	18 500	3.4	4.0
Beckman				
eCAP SDS 200	3400	2400	2.4	1.1
eCAP SDS 14-200	3100	8300	2.8	3.0

short life time (average life time: *ca.* 40 injections) [9].

3.2. Analysis of proteins

A typical electropherogram of rbSt obtained by the ABI and the Beckman replaceable polymer-filled capillary systems are shown in Figs. 2 and 3. Both of these systems achieved base-line resolution of the monomer and dimer of rbSt. Typical composite electropherograms of a polyclonal IgG protein (ATGAM) analyzed by the ABI polymer-filled capillary system are shown in Fig. 4. Due to microheterogeneity of the polyclonal antibody, the major IgG peak was not very sharp. However, both the ABI and Beckman systems clearly resolved the L- and H-chains of the IgG in their reduced state.

Although the presence of the α- and the β-subunits of hemoglobin could be shown when the detector sensitivity was increased (data not shown), none of the SDS polyacrylamide gel- nor polymer-filled capillary systems examined was capable of resolving the subunits, which differ by

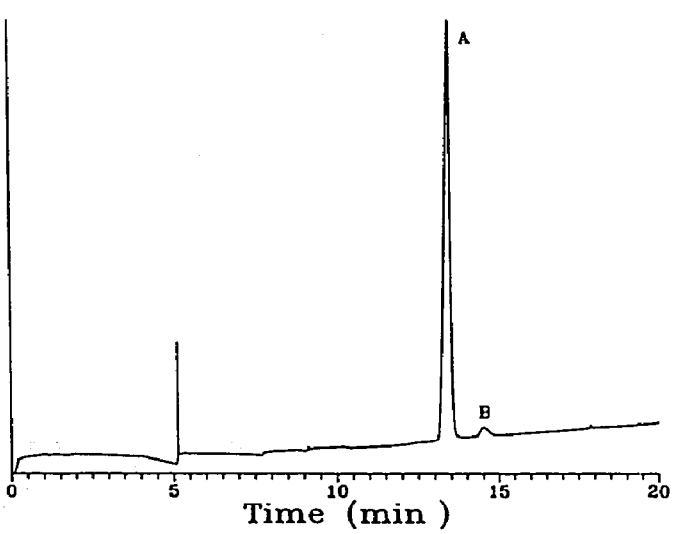

Fig. 2. Electropherogram of rbSt obtained by the Beckman SDS eCAP 200 replaceable polymer-filled capillary electrophoresis system [16]. Peak identification: A = monomer, B = dimer. Conditions, UV at 214 nm; −300 V/cm; column temperature: 20°C; effective peak migration distance: 40 cm; coated capillary: 100 μm I.D.; running buffer: SDS polymer solution from Beckman.

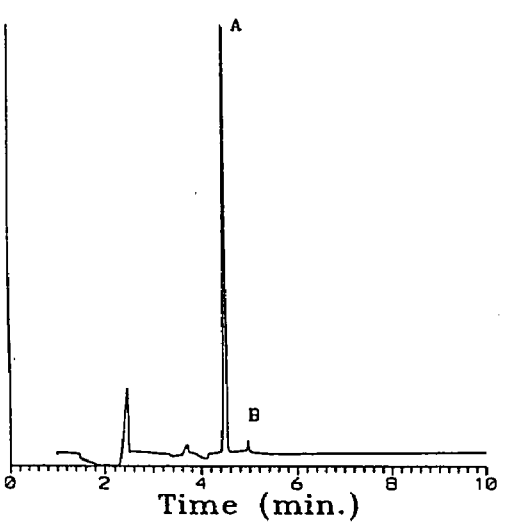

Fig. 3. Electropherogram of rbSt obtained by the ABI replaceable SDS polymer-filled capillary electrophoresis system [17]. Peak identification: A = monomer, B = dimer. Conditions, UV at 214 nm; −300 V/cm; detector: 214 nm; column temperature: 30°C; migration distance: 20 cm; bare fused-silica capillary: 50 μm I.D.; running buffer: SDS polymer solution (ABI).

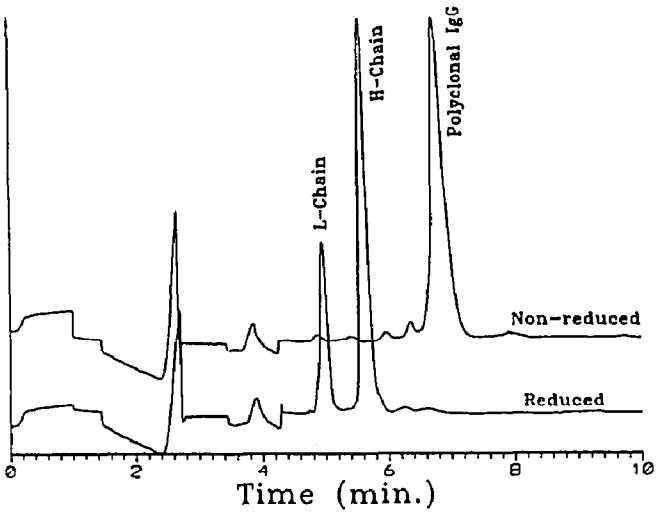

Fig. 4. Replaceable SDS polymer-filled capillary electropherogram of a reduced and a non-reduced sample of a polyclonal IgG (ATGAM) as monitored at 214 nm indicating resolution of L- and H-chains and the intact IgG molecule. Conditions: −300 V/cm; detector: 214 nm; column temperature: 30°C; migration distance: 20 cm; bare fused-silica capillary: 50 μm I.D.; running buffer: SDS polymer solution (ABI).

approximately 800 Da. The minimum peak resolution of the SDS crosslinked polyacrylamide gel-filled capillary electrophoresis system was estimated to be *ca.* 1500 Da [9].

Sensitivity of the polyacrylamide gel- and polymer-filled capillary electrophoresis systems was judged to be approximately equivalent to that of SDS-PAGE, stained with coomassie brilliant blue.

Precision

The precision of the replaceable SDS polymer-filled capillary electrophoresis (rPGCE) system for the determination of the molecular mass of rbSt was examined and is presented in Table 2. A precision (relative standard deviation, R.S.D.) of approximately 1% was obtained for both the peak migration time and the molecular mass for the ABI and Beckman eCAP 200 systems (Table 2). Precision of the Beckman SDS eCAP 14-200 system was similar (R.S.D. 1.2%).

Precision (R.S.D.) of the SDS-PAGE for the determination of the molecular mass of the rbSt was evaluated by scanning a SDS-PAGE slab-gel plate 8 times with a densitometer. A nearly equivalent R.S.D. of 2.0% was obtained (Table 2).

Molecular mass determination

Molecular mass values of proteins determined by the replaceable SDS polymer-filled capillary electrophoresis systems from ABI and Beckman were compared with those analyzed by SDS-

Table 2
Precision of the replaceable SDS polymer-filled capillary electrophoresis systems for the determination of molecular mass of rbSt

	ABI polymer system[a]		Beckman polymer system[b]		SDS-PAGE[c] Molecular mass (Da)
	Peak migration time (min)	Molecular mass (Da)	Peak migration time (min)	Molecular mass (Da)	
Ave.	4.64	20 533	12.80	21 070	21 300
R.S.D.%	0.18	0.90	0.3	1.9	2.0

Theoretical mass value of rbSt: 21 812.

[a] Conditions of the ABI system: −300 V/cm; column temperature: 30°C; effective peak migration distance: 20 cm; bare fused-silica capillary: 50 μm I.D.; running buffer: SDS polymer solution from ABI [17].

[b] Beckman's SDS eCAP 200 system. Condition: −300 V/cm; column temperature: 20°C; effective peak migration distance: 40 cm; coated capillary: 100 μm I.D.; running buffer: SDS polymer solution from Beckman [16].

[c] A SDS-PAGE slab-gel plate was scanned with a densitometer and the molecular mass value was calculated using the ImageQuant software.

PAGE and the HPSEC (Table 3). Although all the SDS polyacrylamide gel- and polymer-filled capillary systems examined separate proteins by molecular sieving [1,12–14], the chemical composition of the commercially available polymer formulations is dissimilar [16,17,22] and is different from the crosslinked polyacrylamide gel.

There was no significant difference between the molecular masses of proteins determined by the replaceable polymer-filled capillary electrophoresis systems from ABI and Beckman (Table 3). In general, the molecular masses obtained closely agreed with those obtained by SDS-PAGE. The molecular masses determined by these SDS systems agreed relatively well with the theoretical values of these proteins (Table 3). The discrepancy between the molecular mass of CD_4-PE as determined by the SDS systems and its theoretical value may be due to the linear, tertiary structure of this molecule [25].

The HPSEC method, which resolves compounds by hydrodynamic radius/volume, gave the least reliable data. The inability of HPSEC to precisely determining the molecular mass of proteins is exemplified by the data on rbSt (Table 3); the duplicate mass data presented were obtained with the same HPSEC method performed by two analysts in two different laboratories. The pH and ionic strength of the mobile phase affect not only the hydrodynamic radius of a protein, but can also cause ionic interaction or hydrophobic binding of the protein to the bonded phase of the HPSEC column [26].

Table 3
Comparison of the molecular mass of proteins determined by the replaceable SDS polymer-filled capillary electrophoretic systems from ABI and Beckman with those of the SDS-PAGE and HPSEC.

Proteins	Molecular mass (Da)						
	SDS polymer-filled capillary		SDS-PAGE	HPSEC		Peak identification	Theoretical values
	ABI[a]	Beckman[b]		lab. A	lab. B		
CD_4-PE	64 500	64 700	68 200 65 600	100 000		monomer:	59 172
rbSt	13 500	15 900	15 800	–	11 900	fragment	
	20 500	21 100	20 400	19 200	36 900	monomer:	21 812
	42 200	45 500	41 800	43 500	80 300	dimer	
	80 800	>62 000		>68 000	113 000	oligomer	
	131 000	–	>126 000				
ATGAM	28 600	24 600	26 000			L-chain:	23 000
	52 000	60 500	51 000			H-chain:	50 000
	43 000	43 500	46 000			Fc fragment:	46 000
	95 300	115 000	98 000			F(ab')2:	100 000
	142 000	152 000	145 000	207 000		IgG:	150 000
Hemoglobin	28 600	24 000	25 800	18 200			
subunit	14 900	14 600	15 700	–		α-subunit:	15 053
			16 300			β-subunit:	15 854

[a] Conditions of the ABI system: −300 V/cm; column temperature: 30°C; effective peak migration distance: 20 cm; bare fused-silica capillary: 50 μm I.D.; running buffer: SDS polymer solution from ABI [17].

[b] Beckman SDS eCAP 200 system. Condition: −300 V/cm; column temperature: 20°C; effective peak migration distance: 40 cm; coated capillary: 100 μm I.D.; running buffer: SDS polymer solution from Beckman [16].

4. Conclusions

The peak resolution capability of the SDS crosslinked polyacrylamide gel-filled capillary electrophoresis system is superior to that of the commercially available replaceable SDS polymer-filled capillary electrophoresis (rPGCE) systems. However, the rPGCE systems enjoy a longer column life time, 140 injections [24] *vs. ca.* 40 injections for the crosslinked polyacrylamide gel [9], and are less prone to the sample carry-over effect, since the polymer matrix is replaced after each assay. Thus, the rPGCE systems have significant advantages over conventional SDS-PAGE by enabling fast, convenient, and automated assay operation and, above all, improved quantitation and reproducibility. The rPGCE system is superior to the HPSEC method for the analysis and the molecular mass determination of proteins of pharmaceutical interest.

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