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Factors affecting the performance of sodium dodecyl sulfate gel-filled capillary electrophoresis

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ABSTRACT

Effects of factors, such as column temperature, internal column diameter, and column length, on the performance of sodium dodecyl sulfate gel-filled capillary electrophoresis were evaluated for the analysis of protein. Increase of column temperature resulted in exponential decrease of peak efficiency. The maximum performance for a 40 cm × 75 μm I.D. column was obtained at the column temperature of *ca.* 21–24°C. Effect of column temperature on peak migration time was minimal. Increase of the internal column diameter resulted in peak migration time to decrease linearly while theoretical plate decreased exponentially. Linear relationship existed between the length of the capillary column and peak migration time and/or theoretical plates.

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 represents a collection of labor-intensive and time consuming techniques and quantification by means of an optical scanner is often sub-optimum. High-performance size-exclusion chromatography (HPSEC) is routinely employed to determine the composition of proteins. However, peak resolution capability of HPSEC is not always ideal [2].

Advances in high-performance capillary electrophoresis (HPCE) instrumentation [3–6] have made it possible to exploit potential of SDS gel-filled capillary systems for separation of proteins with promises of rapid and automated analysis with improved reproducibility and quantification. Hjertén [7] was the first to utilize a polyacrylamide gel-filled capillary column to demonstrate separation of a membrane protein. Cohen and Karger [8] applied SDS polyacrylamide gel-filled capillaries for electrophoresis of peptides and proteins. Neither publications presented quantitative data. Tsuji prepared SDS polyacrylamide gel-filled capillary columns and provided data for molecular mass separation and quantification of recombinant proteins [9].

Zhu *et al.* [10] expanded molecular sieving action of non-acrylamide polymers, *e.g.* dextran, methylcellulose, and polyethylene glycol, and used them as additives in the HPCE buffer to facilitate separation of DNA and proteins [11]. Karger *et al.* [12] also examined a branched dextran and a linear polyethylene glycol polymer networks and successfully achieved separation of proteins. Bode demonstrated sieving effect of a linear polyacrylamide for separation of RNA and proteins based on their molecular masses [13,14]. Widhalm *et al.* [15] used a non-derivatized fused-silica capillary column and applied the linear polyacrylamide for electrophoresis of proteins. Since no attempt was made to eliminate the electroosmotic flow, data presented were preliminary. Just recently such non-cross-linked and/or non-polyacrylamide based gel-filled capillary systems became commercially available

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[16,17]. This paper examines factors affecting performance of such SDS gel-filled capillary electrophoresis systems.

EXPERIMENTAL

Instrumentation

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

Non-acrylamide gel-filled capillary system from Beckman [16]. Each analytical run consists of rinsing a coated capillary column (part No. 241521, Beckman; 100 μm I.D. × 375 μm O.D.; effective length, 40 cm) with 1.0 *M* HCl for 2 min and the column was filled with an SDS non-acrylamide gel solution (part No. 241522, Beckman) for 4 min. Use of a coated capillary column is required to eliminate electroendosmosis.

The column temperature was maintained at 20°C by a circulating coolant to minimize band diffusion for effective size separation. An electrophoretic run was conducted at −300 V/cm (24 μA) using the SDS non-acrylamide gel solution (Beckman) as reservoirs at both anode and cathode terminals. About 1 mg protein per ml solution was injected for 60 s under nitrogen pressure (total injection volume, *ca.* 60 nl) onto the SDS non-acrylamide gel-filled capillary column.

SDS gel-filled capillary system from ABI [17]. A bare fused-silica capillary column (75 μm I.D. × 375 μm O.D.; effective length, 40 cm, Polymicro Technologies, Phoenix, AZ, USA) was activated by a 0.1 *M* NaOH solution for 5 min. Analytical run starts by coating the capillary with the SDS gel solution (parts No. 401482, Applied Biosystems, (ABI), Foster City, CA, USA) for 20 min. Since the gel formulation contains a linear polymer of acrylamide [17] to minimize an electroendosmotic flow, use of a specially coated capillary column is not required. After a couple of electrophoretic runs, the capillary was rinsed and regenerated for 5 min with 1 *M* HCl followed by 0.1 *M* NaOH for another 5 min.

The column temperature was maintained at 20°C by a circulating coolant. Each electrophoretic run was conducted at −300 V/cm (37 μA) using the SDS gel solution (ABI) as reservoirs at both anode and cathode terminals. A sample solution containing about 100 μg protein per ml was injected for 40 s under nitrogen pressure (total injection volume, *ca.* 6 nl) onto the SDS gel-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).

Reagents

The molecular mass protein standard solution containing hen egg white lysozyme (molecular mass 14 400), 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) was obtained from Bio-Rad (No. 161-0304, SDS-PAGE low range molecular mass standard).

Sample buffer solution, containing 1% SDS (Sigma, St. Louis, MO, USA) in 0.12 M Tris–HCl, pH 6.6 (part No. 241525, Beckman), was used to dilute the protein sample. The 2-mercaptoethanol (Sigma) was used to reduce the protein sample.

Preparation of protein samples

Molecular mass protein standard. A 5-μl quantity of the molecular mass protein standard solution (Bio-Rad) was diluted in 40 μl of the sample buffer solution. After through mixing, 2 μl of 2-mercaptoethanol was added and the mixture was heated at 80°C for 5 min.

Protein samples were diluted in the sample buffer solution to approximately 1 mg/ml concentration. To reduce and denature the protein, 2 μl of 2-mercaptoethanol was pipetted into a protein solution. After through mixing, the sample was heated at 80°C for 5 min.

RESULTS AND DISCUSSION

In the presence of SDS, protein forms a SDS–protein complex with a constant SDS–protein ration of 1.4 g/g [1]. Since the SDS gel-filled

capillary system utilizes means to eliminate electroosmotic flow, separation of SDS–protein complexes is strictly based on their size.

Factors affecting performance of SDS gel-filled capillary column

Column temperature. Effects of column temperature on the performance of the SDS gel-filled capillary columns were evaluated using the gel solutions obtained from ABI and Beckman. Effect of column temperature on peak migration time was minimal Fig. 1(I,II). Increase of the column temperature resulted in decrease of the peak migration time of phosphorylase *b*, for example, by approximately $1\%°C^{-1}$ [Fig. 1(I,II)].

Effects of column temperature on the performance/theoretical plate of the gel-filled capillary columns were then evaluated. For the Beckman's system, increase of the column temperature resulted in exponential decrease of theoretical plate (Fig. 2). Theoretical plate of the lysozyme and the carbonic anhydrase peaks, for example, decreased from about 80 000 to 25 000 and about 45 000 to 16 000 by increase of the column temperature from 18 to 30°C, respectively.

According to Jorgenson and Lukacs [19], once thermal equilibrium is established in the capillary, parabolic temperature gradient forms across the radius of the column resulting band broadening. Thus, as the column temperature rises loss of theoretical plates would result. Guttman *et al.* [20] presented a mathematical relationship between the column temperature and efficiency of the Beckman's gel-filled capillary column.

Effects of column temperature on theoretical plate of the ABI's system were complex (Figs. 3 and 4). For a 20 cm × 50 μm I.D. column, increase of the column temperature resulted in slow, yet exponential decrease of theoretical plates (Fig. 3). For example, theoretical plates of the lysozyme peak decreased gradually from about 150 000 to 110 000 by 12°C increase of the column temperature (from 19–33°C.). However, a 40 cm × 75 μm I.D. column behaved differently (Fig. 4). Theoretical plate of the carbonic anhydrase, lysozyme and phosphorylase *b* peaks first increased by increase of the column temperature form 19–20°C, then reached a plateau at the column temperature of *ca.* 21–23°C. This phenomenon, observed with a 40 cm × 75 μm I.D. column, of the theoretical plate to decrease by the decease of the column temperature from 20 to 18°C was confirmed by use of two other columns with the identical dimensions. Explana-

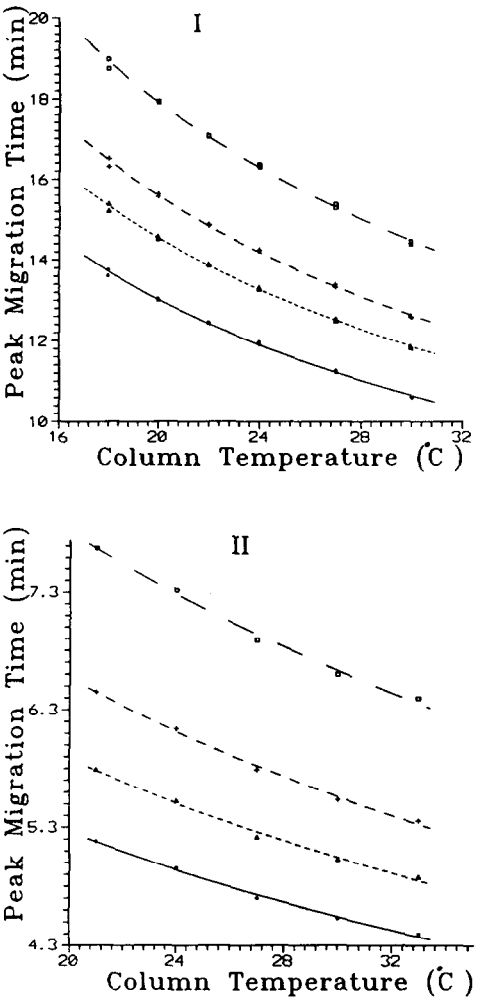

Fig. 1. Effect of column temperature on peak migration of proteins by the SDS gel-filled capillary systems of (I) Beckman and (II) ABI, indicating decrease of peak migration time by increase of column temperature. Conditions: (I) −300 V/cm (24 μA); column temperature: 20°C; effective peak migration distance: 40 cm; coated capillary: 100 μm I.D.; running buffer: SDS non-acrylamide gel solution (Beckman). (II) −300 V/cm (20 μA); column temperature: 30°C; effective peak migration distance: 20 cm; bare fused-silica capillary: 50 μm I.D.; running buffer: SDS gel solution (ABI). □ = Phosphorylase *b*; + = ovalbumin; △ = carbonic anhydrase; ● = lysozyme.

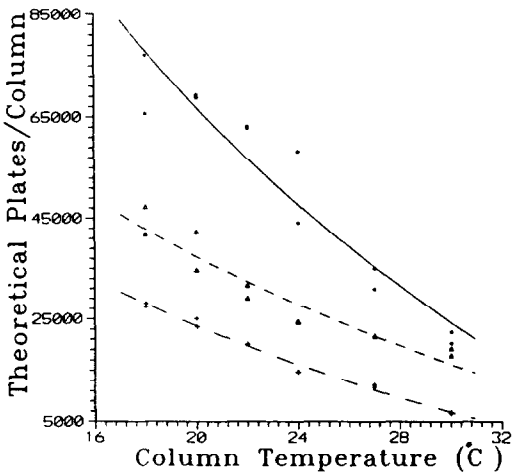

Fig. 2. Effect of column temperature on theoretical plate of the SDS non-acrylamide gel-filled capillary column (coated by Beckman, 40 cm × 100 μm I.D.) indicating exponential decrease of the theoretical plate by the increase of column temperature. Conditions: −300 V/cm (24 μA); column temperature: 20°C; running buffer: SDS non-acrylamide gel solution (Beckman). ● = Lysozyme; △ = carbonic anhydrase; + = phosphorylase *b*.

tion on the phenomenon is not possible without knowing the exact composition of the proprietary gel formulation of the ABI.

Increase of the column temperature beyond

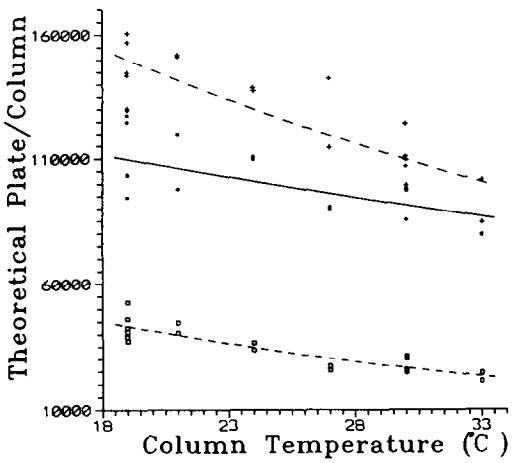

Fig. 3. Effect of column temperature on theoretical plate using a 20 cm × 50 μm bare fused-silica capillary column filled with the SDS gel from ABI indicating gradual decrease of theoretical plate by increase of column temperature. Conditions: −300 V/cm (20 μA); column temperature: 19 to 33°C; running buffer: SDS gel solution (ABI). + = Carbonic anhydrase; ● = lysozyme; □ = BSA.

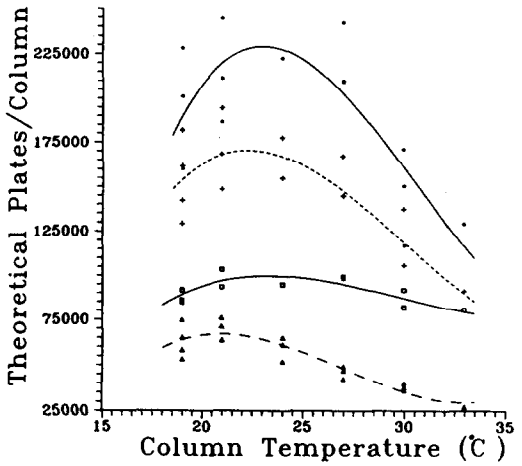

Fig. 4. Effect of column temperature on theoretical plate using a 40 cm × 75 μm bare fused-silica capillary column filled with the SDS gel from ABI indicating the optimum column temperature of 21–23°C for performance of the column. Conditions: −300 V/cm (32 μA); column temperature: 19 to 33°C; running buffer: SDS gel solute (ABI). ● = Carbonic anhydrase; + = lysozyme; □ = trypin inhibitor; △ = phosphorylase *b*.

25°C resulted in theoretical plate of these three peaks to decrease from about 225 000 to 125 000, 170 000 to 90 000, and 65 000 to 25 000, respectively (Fig. 4). The BSA peak behaved similarly.

Effect of column temperature on theoretical plate of the trypsin inhibitor peak was minimal (Fig. 3). The trypsin inhibitor used contained two unresolved peaks of nearly equal proportions. Incomplete resolution of peaks resulted in unreliable theoretical plate numbers. Under such circumstances, minor trend of theoretical plates *vs.* column temperature relationship may not be detected.

Guttman *et al.* [20] evaluated influence of column temperature on the dextran and polyethylene oxide based gel-filled capillary systems. They observed that increase of column temperature resulted in decrease of peak efficiency in the polyethylene oxide based gel system while increase of peak efficiency was noted in the dextran based gel.

Evaluation of column diameter and length. Since the gel formulation of ABI contains a linear polymer of acrylamide [17] to minimize an electroendosmotic flow, use of a specially coated capillary column is not required

to separate proteins based on their molecular masses. Thus, the ABI's gel solution was utilized to evaluate effects of column diameter and column length on the performance of the gel-filled capillary columns.

I. Internal column diameter. 20 cm long columns with an internal diameter (I.D.) ranging from 50 to 150 μm (350 μm O.D.) were used to evaluate effect of internal column diameter on performance of the capillary columns. Experiments were conducted at the column temperature of 20°C with the constant electric potential gradient of −300 V/cm.

As shown in Fig. 5(I), increase of the internal column diameter resulted in exponential decrease of theoretical plate. Theoretical plate of the carbonic anhydrase peak, for example, decreased from about 115 000 to 7 000 by increase of the internal column diameter from 50 to 150 μm, respectively.

Loss of peak efficiency by the increase of the internal column diameter may be attributable to the Joule heating. Column radius significantly affects temperature differential within the capillary column, hence on the column performance. Indeed, effect of column radius on temperature differential, ΔT, was expressed by Cohen *et al.* [18] as

$$\Delta T = (0.24\ Wr^2)/(4k)$$

where: W is the generated power ($W = I^2R$), r is radius of the capillary column, and k is thermal conductivity of the medium. Thus, ΔT is a function of r^2. Effect of column diameter on theoretical plate height (H) was given by

$$H = [7 \cdot 10^{-9} \varepsilon_r \varepsilon_0 \zeta \lambda^2 \varepsilon^2 d_c^6 E^5 c^2]/(D_m \eta K^2)$$

(refer to the ref. 21) where; d_c is a diameter of the capillary, E is an electric potential gradient, and c is the electrolyte concentration. Under the current experimental condition, ε_r, ε_0, ζ, λ, ε, d_c, E, c, D_m, η, and K but not d_c are all constant. Thus, the H is a function of d_c^6. Since $N = 1/H$, N is proportional to $1/d_c^6$. Thus, two equations clearly indicate significant contribution of the column diameter on ΔT and N. For example, when the internal column diameter increases from 50 μm to 100μm, ΔT is expected to increase by 4 times and N decreases by 64 fold.

Although the effects of column diameter on peak migration time was not pronounced, migration time of the carbonic anhydrase peak showed a trend to decrease linearly from 4.8 to 4.2 minutes by increase of the internal diameter from 50 to 150 μm [Fig. 5(II)]. Peak migration time on five other proteins tested, lysozyme, trypsin inhibitor, ovalbumin, BSA, and phosphorylase *b*, behaved similarly.

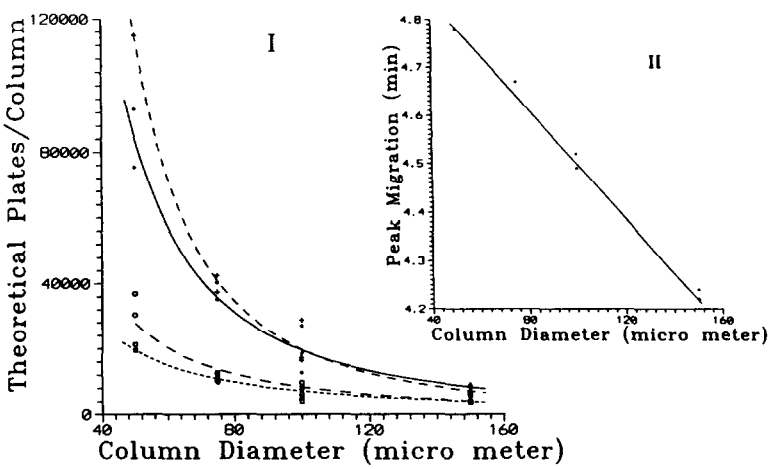

Fig. 5. Effects of column diameter on (I) theoretical plate of the carbonic anhydrase, lysozyme, BSA, and ovalbumin peaks and (II) peak migration time of carbonic anhydrase, using 20 cm long bare fused-silica capillary columns (50 to 150 μm I.D.) filled with the SDS gel from ABI. Conditions: −300 V/cm (*ca.* 20–37 μA); column temperature: 20°C; running buffer: SDS gel solution (ABI). ● = Lysozyme; + = carbonic anhydrase; □ = ovalbumin; ○ = BSA.

Use of a capillary column with an internal diameter smaller than 50 μm would be impractical since it requires longer than 90 min to effectively coat the bare fused-silica capillary column with the gel solution for a successful electrophoretic run.

II. Column length. Experiment was conducted using 75 and 100 μm I.D. columns at the column temperature of 20°C. Electrophoretic run was made at the constant electric potential gradient (E) of −300 V/cm.

For a 100 μm I.D. column, a linear relationship existed between the peak migration time and the column length. Migration time of the phosphorylase *b* peak, for example, increased from about 7 to 28 min by increase of the column length from 20 to 80 cm (Fig. 6). Similar linear relationship between the peak migration time and the column length was observed by the 75 μm I.D. column.

Since the experiment was conducted under the constant electric potential gradient (E) of −300 V/cm ($E = V/L$), the equation of Jorgenson and Lukacs [22] for the expression of peak migration time t_m of

$$t_m = L^2/(V\mu)$$

becomes

$$t_m = L/(E\mu)$$

where: t_m is the solute migration time, L is the column length, V is the voltage, and μ is the solute mobility. Thus, under the current experimental conditions, the relationship between the peak migration time and the column length is linear.

Relationship between the theoretical plate and the length of the column has been expressed as

$$N = (L\mu)/2D_m$$

(refer to ref. 21) where: L is the column length, μ is the solute mobility, and D_m is the diffusion coefficient. Under the current experimental conditions, both μ and D_m are constant. Thus, existence of a linear relationship between the theoretical plate and the column length is indicated. Indeed, for a 75 μm I.D. column, theoretical plates of protein peaks increased linearly by increase of the column length (Fig. 7); theoret-

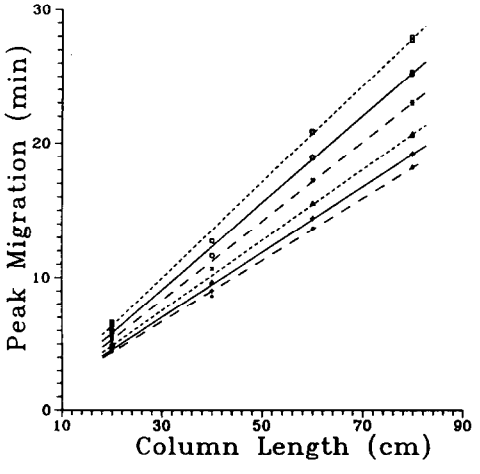

Fig. 6. Effect of column length on peak migration time of proteins using 100 μm I.D. bare fused-silica capillary columns filled with the SDS gel from ABI indicating existence of a linear relationship between the column length and the peak migration time. Conditions: −300 V/cm (20 μA); column temperature: 20°C; effective peak migration distance: 20 to 80 cm; running buffer: SDS gel solution (ABI). □ = Phosphorylase *b*; ○ = BSA; × = ovalbumin; △ = carbonic anhydrase; + = trypsin inhibitor; ● = lysozyme.

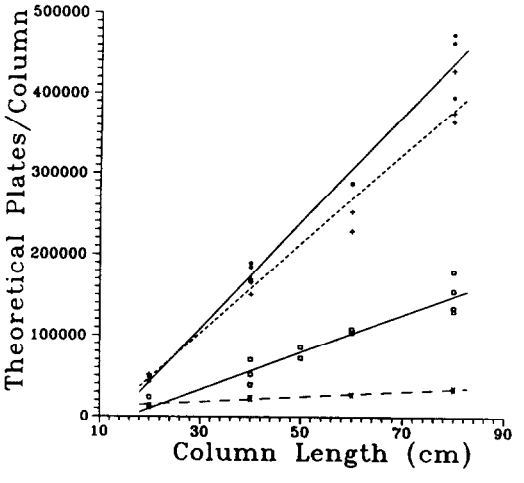

Fig. 7. Effect of column length on theoretical plate numbers of carbonic anhydrase, lysozyme, phosphorylase *b*, and ovalbumin peaks using 75 μm I.D. bare fused-silica capillary columns filled with the SDS gel from ABI indicating existence of a linear relationship between the column length and theoretical plates. Conditions: −300 V/cm (37 μA); column temperature: 20°C; effective peak migration distance: 20 to 80 cm; running buffer: SDS gel solution (ABI). * = Carbonic anhydrase; + = lysozyme; □ = phosphorylase *b*; × = ovalbumin.

ical plates of the carbonic anhydrase peak, for example, increased from about 50 000 to 440 000 by increase of the column length from 20 to 80 cm. However, increase of the theoretical plate was non-linear by use of various lengths of the 100 μm I.D. column (Fig. 8). Increase of theoretical plates of the lysozyme, trypsin inhibitor, and ovalbumin peaks reached a plateau at the column length of *ca.* 60–70 cm (Fig. 8). Non-linear relationship between the theoretical plates and the column length observed for the 100 μm I.D. column may be due to increased Joule heating.

Rs is a factor that is also expected to increase by increase of the column length. *Rs* is given by

$$Rs = 0.177(\mu_1 - \mu_2)[V/(D\mu_{av})]^{1/2}$$

(refer to the ref. 19). Since $E = V/L$, *Rs* can be expressed as

$$Rs = 0.177(\mu_1 - \mu_2)[EL)/(D\mu_{av})]^{1/2}$$

where: μ_1 and μ_2 are mobility and μ_{av} an average mobility of the 2 solutes 1 and 2, V is voltage, and D is the diffusion coefficient. The μ can be expressed as

$$\mu = (\varepsilon\zeta)/\eta$$

(refer to ref. 20) where: ε, ζ, and η are dielectric constant of the medium, zeta potential, and viscosity of the medium, respectively. Under the current experimental conditions, μ, E, and D are constant. Thus, *Rs* is a function of the square root of L. The *Rs* between the carbonic anhydrase and the ovalbumin peaks, obtained by use of a 20, 40, 60 and 80 cm long, 75 μm I.D., columns at the column temperature of 20°C, were 3.65, 5.73, 6.44, and 7.30, respectively. Indeed, these *Rs* values are in close agreement (correlation coefficient: 0.98) with those of 3.65, 5.16, 6.32 and 7.43, predicted form the equation.

Use of a 40 cm × 50 μm I.D. column was also attempted. However, in spite of over 90 min of the column coating operation, no migration of protein peak was detected. As the inner column surface increases, operation to effectively coat the bare fused-silica capillary column by use of the gel-solution from ABI became progressively difficult.

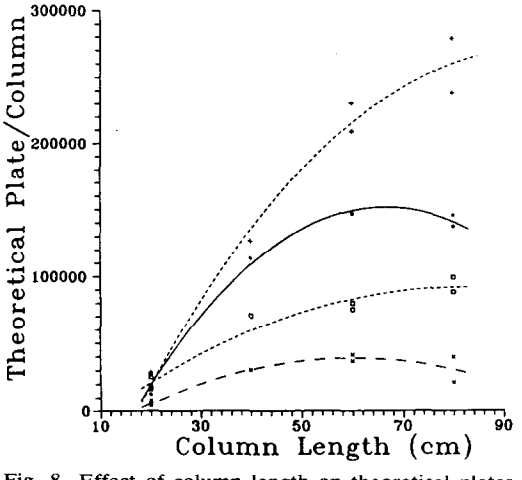

Fig. 8. Effect of column length on theoretical plates of the carbonic anhydrase, lysozyme, trypsin inhibitor, and ovalbumin peaks using 100 μm I.D. bare fused-silica capillary columns filled with the SDS gel from ABI indicating that theoretical plate number reaches a plateau at the column length of *ca.* 60–70 cm due to the Joule heating. Conditions: −300 V/cm (20 μA); column temperature: 20°C; effective peak migration distance: 20 to 80 cm; running buffer: SDS gel solution (ABI). + = Carbonic anhydrase; ● = lysozyme; □ = trypsin inhibitor; × = ovalbumin.

Practical consideration

In order for any assay method to be adopted by the pharmaceutical quality control laboratories, the method must improve quality of the assay results (*e.g.* precision and accuracy of the molecular mass determination) and/or productivity of the laboratory operation (*e.g.* speed and ease of the assay).

At the column temperature of 20°C, peak migration time of phosphorylase *b* (the largest molecular mass among the proteins examined) was approximately 8, 16, and 16 min for 20 cm × 50 μm I.D., 40 cm × 75 μm I.D., and 40 cm × 100 μm I.D. columns, respectively. It took about 40, 20, and 20 min to effectively coat these bare fused-silica capillary columns with the gel solution. After a couple of assay operations, the capillary column must be stripped and regenerated with HCl and NaOH solutions. Thus, cumulatively, each one assay operation took approximately 60, 45, and 45 min by these three columns, respectively.

Theoretical plate numbers of approximately

90 000, 1650 000, and 80 000 for the lysozyme peak were obtained by use of 20 cm × 50 μm I.D., 40 cm × 75 μm I.D., and 40 cm × 100 μm I.D. columns, respectively. Theoretical plates of the carbonic anhydrase peak were about 115 000, 450 000, and 250 000 for these three columns, respectively. Thus, the 40 cm × 75 μm I.D. column consistently outperformed the other two columns examined. The 75 μm I.D. column also gave a reasonable assay operation time and thus would be the SDS gel-filled column of choice for the assay of proteins. If critical analysis of a protein sample is required and one can afford a longer analysis time, then use of a longer capillary column to obtain an additional resolution may be justified. Development of a mathematical model to optimize column parameters, such as temperature, diameter, and length for the performance of the gel-filled capillary column is being contemplated.

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