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RAPID ANALYTICAL GEL CHROMATOGRAPHY OF PROTEINS AND PEPTIDES ON SEPHADEX MICROBORE COLUMNS

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SUMMARY

A method has been developed for the rapid analytical gel chromatography of low-molecular-weight proteins and peptides on microbore columns (3 mm I.D.) packed with Sephadex G-50 and G-25. The time of a complete chromatographic analysis can be as short as 30 min using only 15–30 μg of protein. The elution parameters of proteins are compared in terms of "retention time" rather than elution volume under conditions of constant flow rate of eluent. The technique has been used successfully for the estimation of the molecular weight of peptides and proteins in the range of 1000 to 26,000 daltons.

INTRODUCTION

The significant contributions of analytical gel filtration to the separation, physical characterization, and interaction of proteins have been well documented[1–4]. However, most of the methods described so far require a considerable amount of material and extensive length of time for the analysis. Thin-layer gel chromatography[1–4] offers some convenience in handling simultaneously multiple samples of microgram quantity, but the method is not quantitative and exhibits other shortcomings[3] which are not encountered in single-column elution experiments. Miniature or capillary column techniques[5–9] appear to be promising in terms of sample size and separation time when applied to micro analysis using liquid chromatography.

The aim of this work was to develop a rapid and quantitative analytical gel chromatography procedure as exemplified in experiments using Sephadex gels (G-50 and G-25) and low-molecular-weight proteins and peptides.

EXPERIMENTAL

Materials

Sephadex G-25 (fine), G-50 (medium), G-100 (medium) and Blue Dextran were obtained from Pharmacia. Chymotrypsinogen A, ribonuclease, cytochrome *c*, and lysozyme were purchased from Calbiochem. Insulin, α-chymotrypsin, and polymixin B were products of Sigma. Bacitracin, thyroglobulin, insulin A, and dinitrophenyl alanine were obtained from Schwarz/Mann. Bovine serum albumin was a product of Pentex. All the other chemicals were of reagent grade.

Gel chromatography

A Chromatronix microbore column (MB-3-500), 2.8 mm bore and 500 mm length, with inlet and outlet Cheminert fittings was used for these experiments. A sample injection tee (Chromatronix, 107B25) was attached directly to the top of the column, by removing the inlet tube and G-Bushing. A silicone rubber septum was used in the top plug and microliter samples were injected with a Hamilton syringe by piercing the septum. This was accomplished by stopping the flow of buffer momentarily but without decreasing the pressure. A Milton Roy instrument mini-pump was used to pump buffer from a reservoir to the inlet of the sample injection tee at a constant flow rate (5 ml/h). The pump was also used to pack the column under positive pressure. In order to avoid excessive back pressure the bed support disc of the microbore column was replaced by a disc made up of four layers of Miracloth (Calbiochem) and two layers of Nylon netting (Pharmacia).

The eluent from the column was monitored at 280 nm with a Gilford recording spectrophotometer (Model 2000) equipped with a Beckman monochromator and Gilford flow cells (10 mm pathlength). The recorder was operated at the constant speed of 15 in./h (0.635 cm/min). An event marker was actuated simultaneously with the injection of the sample.

Sephadex G-25, G-50, and G-100 equilibrated with pH 7.6 phosphate buffer (0.1 *M* in NaCl) was used as the column packing material.

Data presentation

Since the flow rate is constant (5 ml/h or 83 μl/min) and the recorder is operated at constant speed (15 in./h or 0.635 cm/min), the elution volume can be calculated from the chart of the recorder, being approx. 131 μl/cm. However, it was considered desirable to express the data in terms of retention time[10] rather than elution volume. A digital clock can then be used directly for data reduction in conjunction with the digital absorbance meter accessory of the Gilford spectrophotometer.

Partition coefficients were determined from the ratio $(t_e - t_0)/t_t$ corresponding to $(V_e - V_0)/V_t$ in volume measurements[3]. The minimum retention time (t_0) for the column was determined by using thyroglobulin or Blue Dextran. The maximum retention time (t_t) was obtained with dinitrophenyl alanine. The retention time of a particular protein or peptide is expressed by t_e. The retention time represents the time interval between introduction of the sample pulse and emergence of its peak concentration.

For molecular weight estimation, the data were plotted according to HJERTÉN[11] substituting $(V_e - V_0)/V_t$ with $(t_e - t_0)/t_t$. The intercept and slope of the straight lines were derived by least squares treatment of the data.

Peak areas were determined by cutting-out tracings of the recording and weighing. The weight of 1 cm^2 area of the paper was used as a unit in converting weights to cm^2-values.

RESULTS AND DISCUSSION

Elution pattern

A typical elution diagram of thyroglobulin (10 μg) and cytochrome *c* (20 μg) on Sephadex G-50 is shown in Fig. 1. This amount of thyroglobulin is adequate

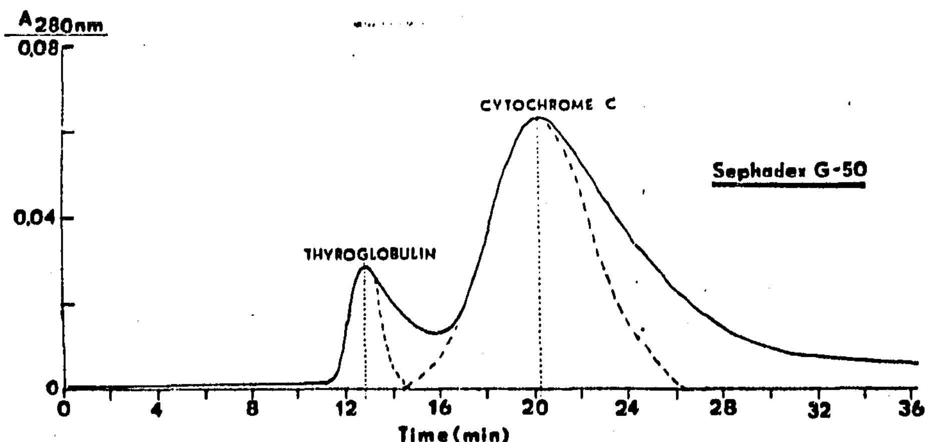

Fig. 1. Example of typical elution profile obtained from the Sephadex G-50 microbore column. Protein sample: thyroglobulin (10 μg) and cytochrome *c* (20 μg); flow rate: 5 ml/h; buffer: pH 7.6 phosphate (0.1 *M* in NaCl). Dotted line indicates hypothetical Gaussian distribution of the peak. Note the trailing rear boundary.

for measurement of the minimum retention time (t_0) of the column and it can be routinely incorporated in the test sample. As it may be seen in the elution pattern, the two major advantages of the method are speed of analysis and sensitivity of detection. A complete gel chromatography experiment can be completed within 30–40 min using only a few micrograms of protein. The major disadvantage of the technique, at its present state of development, is that the peaks exhibit a trailing rear boundary. This is probably due to "wall effects", and it is possible that it could be eliminated by using columns specially designed to disrupt flow along the wall.

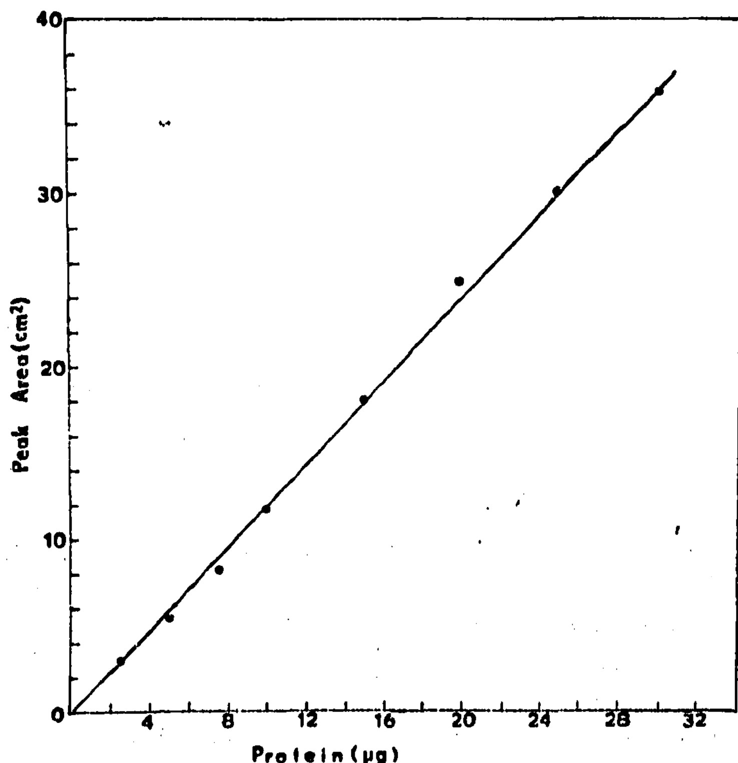

Fig. 2. Linear relationship between elution peak area and amount of lysozyme applied to a Sephadex G-50 column.

An example of the construction of such columns (10.5 mm diameter) has been given by HANSON[12]. Coating of the column with dimethyldichlorosilane[12] (Bio-Rad) resulted only in minor improvement of peak distribution.

Sensitivity of detection

In order to establish the sensitivity of detection of the method, microgram amounts of lysozyme were subjected to gel chromatography on Sephadex G-50 and the areas of the resulting peaks were estimated. Fig. 2 shows that as little as 2.5 μg of lysozyme could be easily detected, and its retention time measured. Also a quantitative relationship was obtained between the amount of protein injected and the corresponding peak area. This suggests that the method could be used for the quantitative determination of proteins in the presence of lower- or higher-molecular-weight UV-absorbing contaminants.

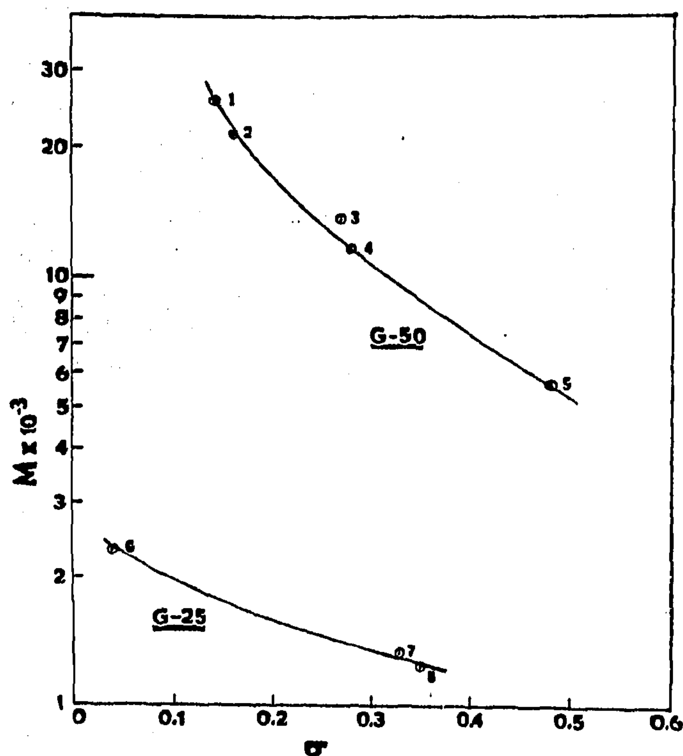

Fig. 3. Plots of partition coefficient (σ) against molecular weight (M) $\times$ 10^{-3} for marker proteins and peptides on Sephadex G-50 and G-25. (1) Chymotrypsinogen; (2) α-chymotrypsin; (3) ribonuclease; (4) cytochrome *c*; (5) insulin; (6) insulin A; (7) bacitracin; (8) polymixin B.

Calibration with marker proteins and peptides

Peptides and proteins of known molecular weight such as polymixin B, bacitracin, insulin A, insulin, cytochrome *c*, ribonuclease, α-chymotrypsin, and chymotrypsinogen A were used as markers in establishing the relationship between partition coefficient (σ) and molecular weight (M). Such plots obtained with the Sephadex G-25 and G-50 columns are shown in Fig. 3.

A graphical presentation of the experimental data according to HJERTÉN[11] is shown in Fig. 4. Plots of $-\log \sigma$ against $M^{2/3} \times 10^{-2}$, where σ represents the partition coefficient $(t_e - t_0)/t_t$ and M the molecular weight, result in straight lines.

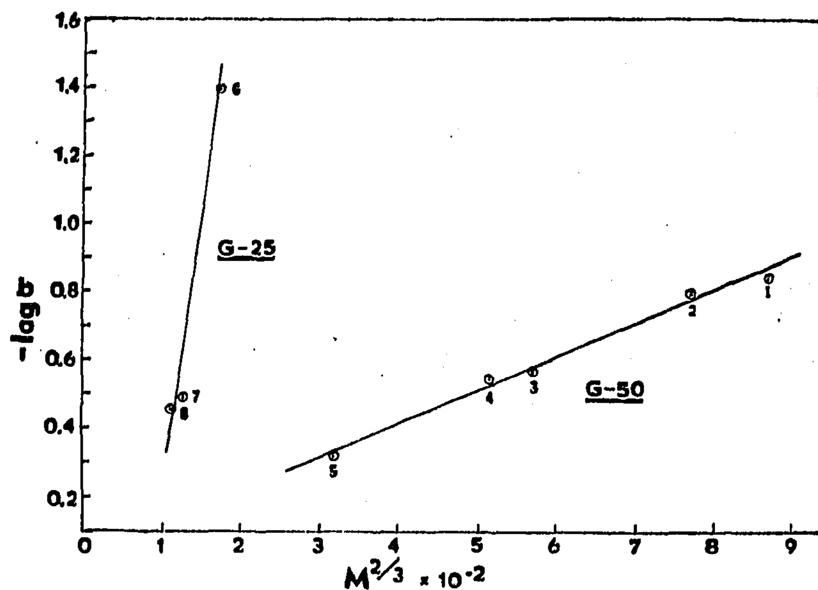

Fig. 4. Plots of $-\log \sigma$ (σ = partition coefficient) against $M^{2/3} \times 10^{-2}$ (M = molecular weight) for marker proteins and peptides from data obtained using Sephadex G-50 and G-25 (see ref. 11). (1) Chymotrypsinogen; (2) α-chymotrypsin; (3) ribonuclease; (4) cytochrome *c*; (5) insulin; (6) insulin A; (7) bacitracin; (8) polymixin B.

Thus, the peptides and proteins used in this study appear to obey the empirical relationship[11]:

$$-\log \sigma = R \times M^{2/3} + S \quad (1)$$

Using regression analysis of the data by the least squares method, the constants R and S were calculated for Sephadex G-25 and G-50. The empirical regression equations obtained were:

$$\text{Sephadex G-25: } -\log \sigma = 0.016270 \times M^{2/3} - 1.458 \quad (2)$$

$$\text{Sephadex G-50: } -\log \sigma = 0.000978 \times M^{2/3} + 0.0214 \quad (3)$$

TABLE I

CORRELATION BETWEEN MOLECULAR WEIGHT (M) AND PARTITION COEFFICIENT (σ) OF PEPTIDES AND PROTEINS ON SEPHADEX G-50 AND G-25

Materials	σ	M[a]	*Apparent M*	*Deviation* (%)
Chymotrypsinogen A	0.14[b]	25,700	24,840[d]	(−) 3.4
α-Chymotrypsin	0.16[b]	21,500	22,290[d]	(+) 3.7
Ribonuclease	0.27[b]	13,700	13,250[d]	(−) 3.3
Cytochrome *c*	0.28[b]	11,700	12,672[d]	(+) 8.3
Insulin	0.48[b]	5,700	5,308[d]	(−) 6.9
Insulin A	0.04[c]	2,300	2,324[e]	(+) 1.1
Bacitracin	0.33[c]	1,400	1,302[e]	(−) 7.0
Polymixin B	0.35[c]	1,200	1,275[e]	(+) 6.3

[a] From amino acid sequence analysis data (see ref. 13).
[b] Obtained with Sephadex G-50.
[c] Obtained with Sephadex G-25.
[d] Calc. from Eqn. (3).
[e] Calc. from Eqn. (2).

Eqns. 2 and 3 were used to calculate the molecular weight of the marker proteins. The results (shown in Table I) indicate that molecular weights of peptides and proteins can be determined with an accuracy of better than 8 % with reproducibility of partition coefficients of approx. 5 %. Considering the sensitivity and speed of analysis of the present method, the obtained values appear to be within reasonable limits.

Experiments with Sephadex G-100

A limited number of experiments were performed using Sephadex G-100. An elution pattern of thyroglobulin and bovine serum albumin mixture is shown in Fig. 5. Although, we have demonstrated that Sephadex G-100 can be used for rapid

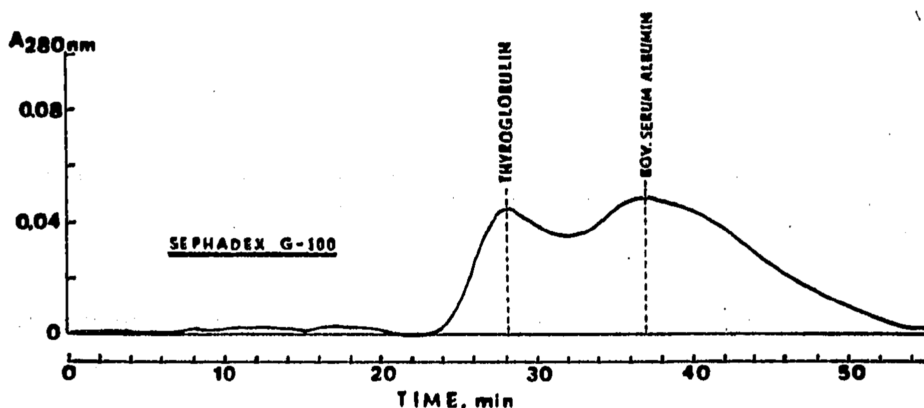

Fig. 5. Elution profile of thyroglobulin (20 μg) and bovine serum albumin (40 μg) from the Sephadex G-100 microbore column. Flow rate: 5 ml/h; buffer: pH 7.6 phosphate (0.1 M in NaCl).

gel filtration experiments, our equipment did not allow a large number of analyses to be performed. This was due to the high pressure developed in the system which resulted in leaks and therefore inaccurate measurements. A high pressure liquid chromatograph will be more suitable for this purpose.

General comments

The advantages offered by the present method are speed of analysis and sensitivity of detection. An automated system may be capable of analysing at least 32 samples per 24 h using microgra mquantities of material. The major disadvantages observed include a trailing rear boundary and development of high pressures with Sephadex G-100 making necessary the use of special equipment. EDWARDS AND HELFT[14] working with compressed Sephadex G-50 gels demonstrated improved resolution by compression and predicted the necessity of using special equipment with the softer gels.

In view of the promising aspects of this technique in the micro analysis of proteins and peptides, further experiments with high pressure liquid chromatography equipment of the effects of gel porosity, flow rate, and compression on resolution and time of analysis are desirable. It is possible that the development of high pressure with the softer gels may affect the conformation of certain proteins[15]. In such a case experiments using 6 M guanidine hydrochloride[16] may be advantageous.

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