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Note

Adsorption chromatography on Bio-Gel P-2*

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In order to resolve a complex mixture of biological compounds present in the ultrafiltrate of patients with chronic uraemia, a gel chromatographic method on polyacrylamide gels (Biogel P-2; Bio-Rad Labs.) has been developed in our laboratories[1]. This method offers better results and reproducibility than those reported by others[2–4].

These results have been explained in terms of an optimization of the experimental conditions: a linear flow-rate lower and a higher ionic strength of the mobile phase than those use by others are believed to be the two factors favourably influencing the separation.

As the substances involved in the separation possess a low molecular weight, other possibilities associated with the partition and adsorption properties of the Bio-Gel P-2 could be considered. Actually, in our experience and in that of other authors[4–7], it was impossible to obtain a calibration graph with Bio-Gel P-2 at any linear flow-rate for molecular weights ranging from 1800 and 100 daltons (the value given by the manufacturer for this gel). It therefore appeared reasonable to assume that, with low-molecular-weight compounds, the partition and adsorption properties of this stationary phase were also important in determining the separation of the mixture.

The adsorption properties of Bio-Gel P-2 towards sulphanilamide, phenol and butanol have been studied[8,9]. Scattered examples of separations of tetracyclines[10], monomeric oxo anions of phosphorus[11] and aromatic molecules[12], presumably based on the affinity properties of this gel phase, have also been described. A systematic study of the limits and possibilities of this stationary phase in the separation of small molecules has not been carried out, however.

In this investigation, a wide variety of small organic molecules have been separated, and it is shown that the separations achieved were not based on molecular weights; a study was made of this unconventional use of a chromatographic column filled with Bio-Gel P-2.

* Dedicated to the memory of Eraldo Antonini prematurely deceased on March 19th, 1983.

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This chromatography, as previously observed for Sephadex LH-20 and G-10[13,14], does not follow the rules of gel chromatography, but appears to be very useful in practical laboratory work. Good reproducibilities and high efficiencies in separating a large variety of compounds can be achieved using very simple apparatus.

EXPERIMENTAL

Bio-Gel P-2 (150–300, 80–150, 40–80 and < 40 μm) was purchased from Bio-Rad Labs., Richmond, CA, U.S.A.; the column was a K 16/100 with thermostatted jacket and two adaptors obtained from Pharmacia (Uppsala, Sweden); the bed height was 83 cm. A peristaltic pump was a Varioperpex from LKB (Stockholm, Sweden).

Elution was performed at a flow-rate of 0.5 ml/min by monitoring at 254 nm with a Uvicord S (LKB) and collection in 1-ml fractions on an UltroRac II fraction collector (LKB).

Analytical-reagent grade products were used. The elution volumes reported are averages of at least three experiments, each differing from the other by about 1%. All the samples were introduced into the column in a 2-ml volume by suction through the peristaltic pump.

RESULTS AND DISCUSSION

Bio-Gel P is a chromatographic support made by cross-linking acrylamide with a bifunctional acrylamide such as N,N'-methylenebisacrylamide.

Bio-Gel P-2 has a declared resolving power as a molecular sieve between 1800 and 100 daltons at optimal linear flow-rates. However, a mixture of calcein, cyanocobalamin, uric acid, phenylalanine and tyrosine (with molecular weights of 622, 1355, 168, 165 and 181 daltons, respectively) is well resolved in a Bio-Gel P-2 column at different linear flow-rates but not according to their molecular weights, reported in Fig. 1.

In Table I elution volumes (V_e) of many organic molecules are reported; it can be seen that the column is able to separate very similar compounds such as 4-nitrobenzoic acid (V_e = 67 ml) and 3-nitrobenzoic acid (V_e = 73 ml). Amino acids such as phenylalanine and tyrosine are also separated.

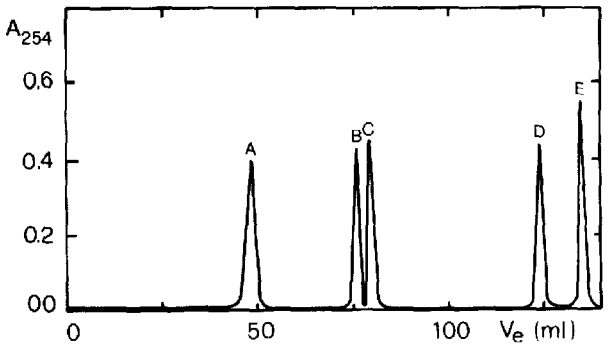


Fig. 1. Elution pattern of a mixture of calcein (A), cyanocobalamin (B), uric acid (C), phenylalanine (D) and tyrosine (E). The molecular weights are 622, 1350, 168, 165 and 181 daltons, respectively. Column, 83 × 1.6 cm I.D., Bio-Gel P-2, 80–150 μm; 50 mM NH_4HCO_3, pH 8; 20°C; flow-rate, 0.5 ml/min.

TABLE I

ELUTION VOLUMES ON A COLUMN (83 × 1.6 cm) OF BIO-GEL P-2

Conditions: particle size, <40 μm; 50 m*M* NH_4HCO_3; pH 8; 20°C.

Sample	*Molecular weight*	V_e *(ml)*	*Sample*	*Molecular weight*	V_e *(ml)*
Insulin	6000	47	Uric acid	168	79
Inulin	5000	47	Phenylalanine	165	124
Calcein	622	47	Tyrosine	181	135
2-Methoxybenzoic acid	152	62	Creatinine	113	159
4-Methoxybenzoic acid	152	64	Methyl benzoate	136	178
Benzoic acid	122	64	Methylguanidine	73	184
1-Chlorobenzoic acid	156	65	Benzaldehyde	106	188
4-Nitrobenzoic acid	167	67	Benzamide	121	188
2-Nitrobenzoic acid	167	68	Tryptophan	204	193
3,5-Diaminobenzoic acid	170	68	Resorcinol	110	210
3-Aminobenzoic acid	137	69	Phenol	94	215
4-Aminobenzoic acid	137	70	Benzene	78	445
2-Aminobenzoic acid	137	72			
3-Nitrobenzoic acid	167	73			
Cyanocobalamin	1355	76			

According to the structure of the stationary phase (*i.e.*, the gel is not penetrable by large molecules), high-molecular-weight compounds are eluted with the void volume of the column. In contrast, small molecules (those lying in the range 100–1800 daltons) can interact with the stationary phase, as they pass through the gel.

Actually, V_e is not strongly influenced by the size of the particles: in adsorption at the surface of the particles, an increase in the elution volumes would be expected with smaller particles. However, this does not appear to occur, as shown in Fig. 2, and this result must be interpreted in terms of interactions inside the gel network.

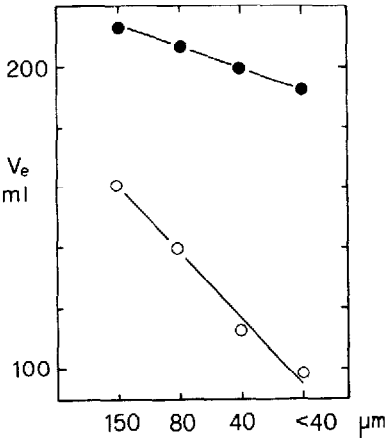


Fig. 2. Elution volumes (V_e) of benzamide (●) and benzoic acid (○) as a function of the diameter (μm) of the particles of the stationary phase (150 = 150–300 μm; 80 = 80–150 μm; 40 = 40–80 μm). Column, 83 × 1.6 cm I.D., Bio-Gel P-2; 50 m*M* NH_4HCO_3, pH 8; 10°C; flow-rate, 0.5 ml/min.

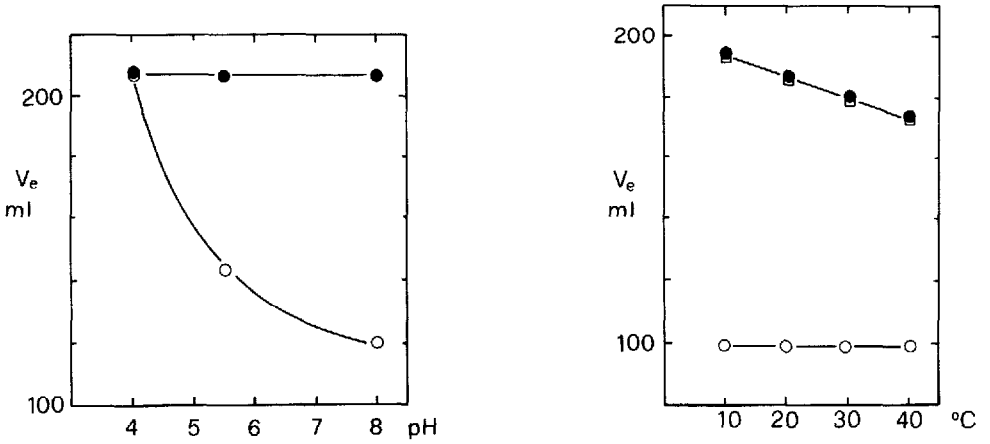


Fig. 3. Elution volumes (V_e) of benzamide (●) and benzoic (○) as a function of pH of the mobile phase. Column, 83 × 1.6 cm I.D., Bio-Gel P-2, 80–150 μm; 10°C; flow-rate, 0.5 ml/min.

Fig. 4. Elution volumes (V_e) as a function of temperature of benzamide (●) and benzoic acid (○) at pH 8 (50 m*M* NH_4HCO_3) and benzoic acid (□) at pH 3.2 (50 m*M* acetic acid). Column, 83 × 1.6 cm I.D., Bio-Gel P-2, <40 μm; flow-rate, 0.5 ml/min.

Many workers have pointed out that these gels, such as the dextran gels, may form either hydrogen or hydrophobic bonds with adsorbates, thus modifying the elution volumes of the molecules passed through a gel chromatographic column[5–9].

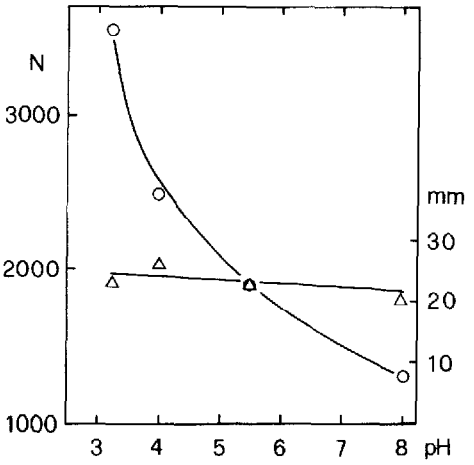
Thermodynamic studies carried out on Bio-Gel P-2 have shown that the adsorbate–adsorbent linkages are first hydrogen bond and then hydrophobic in nature[8,9]. Then the mass of the adsorbent exerts an important role in the separation of two molecules having similar elution volumes. As an example, a column with a bed height of 83 cm and I.D. 1.6 cm (as reported in Table I) is able to take advantage of the differences in elution volumes due to the different partition and adsorbing properties of the gel, to obtain a satisfactory separation between two molecules even when they are very similar. A short column (12.5 cm) does not give an effective separation because the adsorbent mass is not sufficient.

In separations with Bio-Gel P-2, the most important role seems to be played by the hydrogen bonds formed between the adsorbate and the gel.

The elution volume of benzamide does not depend on the pH, as reported in Fig. 3, whereas V_e for benzoic acid decreases as the pH increases. The faster elution of ionizable compared with neutral molecules is presumably related in part to the presence in the polyacrylamide gels, as for dextran gels, of carboxylic groups[15].

Actually, the data in Fig. 4 suggest that the adsorption phenomena given by benzamide might be related to hydrogen bonds, as can be inferred from the strong dependence of V_e on temperature, whereas the interaction of ionized benzoic acid definitely has a different origin, not being dependent on temperature. On the other hand, the elution volume of benzoic acid is itself influenced by temperature when the chromatography is performed at pH 3.2, where the acid is largely undissociated. Under these conditions the behaviour of benzoic acid becames identical with that of benzamide, as shown in Fig. 4.

The large elution volume of benzene is not surprising. Benzene has a very poor



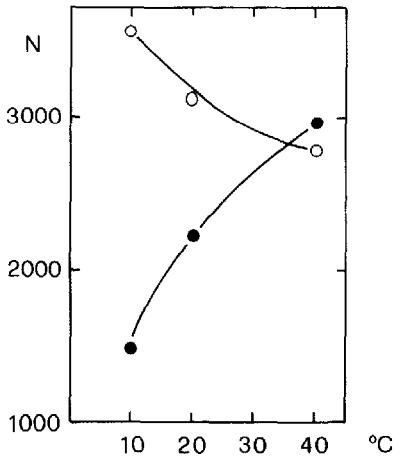


Fig. 5. Number of theoretical plates, N (left-hand ordinate, ○) and peak width (right-hand ordinate, △) in mm for benzoic acid of a column (83 × 1.6 cm I.D.) of Bio-Gel P-2, <40 μm, as a function of the mobile phase. Elution was performed at a flow-rate of 0.5 ml/min at 10°C.

Fig. 6. Number of theoretical plates, N, for benzoic acid of a column (83 × 1.6 cm I.D.) of Bio-Gel P-2, <40 μm, as a function of temperature. Elution was performed at a flow-rate of 0.5 ml/min in 50 mM NH_4HCO_3 at pH 8 (●) and in 50 mM acetic acid at pH 3.2 (○).

affinity for water and can strongly interact with the stationary phase through hydrophobic bonds and/or hydrogen bonds. Accordingly, phenol and resorcinol, which are more soluble in water, have lower elution volumes than benzene although they have stronger absolute interactions than benzene with the stationary phase.

It is interesting that the number of theoretical plates, N, of the column used is rather high, ranging from 1000 to 3500 depending on the experimental conditions that were investigated for benzoic acid. As expected, N decreases with increasing pH of the eluent, as shown in Fig. 5: in fact, an increase in pH increases the percentage of the ionized form of benzoic acid, which further interacts with the gel matrix, giving a lower value of N for the system. It should be noted, however, that the peak width is almost invariant. The fact that the interactions between benzoic acid and the gel matrix are different in nature according to the pH of the mobile phase is apparent also from Fig. 6.

The number of theoretical plates increases with increasing temperature at pH 8 (hydrophobic interactions between ionized benzoic acid and the gel), but decreases with increase in temperature at pH 3.2 (hydrogen bonds).

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