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LIQUID CHROMATOGRAPHY OF NUCLEIC ACID COMPONENTS AND THEIR ANALOGUES ON HYDROXYETHYL METHACRYLATE GELS

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SUMMARY

Spheron hydroxyethyl methacrylate gels are advantageous sorbents for the high-performance liquid chromatography of various nucleic acid components and their analogues. In aqueous media these compounds are reversibly sorbed on the surface of the gel. Differences in the sorption characteristics of particular derivatives enable good separations of even relatively complicated mixtures to be achieved.

INTRODUCTION

Spheron hydrophilic macroporous gels, prepared by copolymerization of hydroxyethyl methacrylate with ethylene dimethacrylate[1], are a new type of sorbent for chromatographic separations. The gel beads show excellent mechanical properties and are able to withstand the high pressures used in modern liquid chromatographic (LC) techniques. Unmodified Spheron gels have been used for chromatographic separations of various natural polymers[1–3], based on molecular sieving[1–3] or a combination of molecular sieving with weak reversible sorption effects[3]. These sorption effects, especially for aromatic and heterocyclic derivatives, also permit the use of Spheron hydroxyethyl methacrylate gels in LC separations of numerous low-molecular-weight compounds. An example of such a use is the separation of various nucleic acid components and their analogues. Interactions of these compounds with the inner surface of Spheron gels resemble those described for tightly cross-linked dextran or polyacrylamide gels[4].

EXPERIMENTAL

Materials

Spheron 100 and 1000 hydroxyethyl methacrylate gels of grain size 25–40 μm, most of the purine and pyrimidine bases, ribonucleosides, 2′-deoxyuridine, 5-iodo-2′-deoxyuridine, 1-(β-D-arabinofuranosyl)uracil, 2,2′-anhydro-1-(β-D-arabinofuranosyl)uracil, adenosine-3′,5′-cyclic phosphate and glucose were products of Lachema (Brno, Czechoslovakia) or were prepared in Research Institute of Pure Chemicals, Lachema. 5-Fluorouracil, 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine,

thymidine and nucleoside-5'-phosphates were products of Calbiochem (San Diego, Calif., U.S.A.). Dextran T 2000 was obtained from Pharmacia (Uppsala, Sweden).

Apparatus

Spheron 100 or 1000 was packed in a stainless-steel column (20 × 0.8 cm). Eluent was pumped with an MC 706-300 piston pump (Mikrotechna, Prague, Czechoslovakia), connected with the column through a pulse damper. For detection and recording of elution profiles, a DUV 254 differential UV detector (Vývojové dílny ČSAV, Prague, Czechoslovakia) attached to an EZ 10 recorder (Laboratorní přístroje, Prague, Czechoslovakia) was used. For the evaluation of the column parameters, a Model 401 differential refractometer (Waters Assoc., Milford, Mass., U.S.A.) was used.

Methods

The column was packed with Spheron 100 or 1000 gel by a modified technique[5]. Samples (10–50 μl) were applied as 0.02–0.1 % solutions of the derivatives in 0.1 *M* sodium phosphate (pH 7). Elution was performed using 0.1 *M* sodium phosphate (pH 7) or, in other series of experiments, 1 *M* sodium chloride in 0.1 *M* sodium phosphate (pH 7), 0.01 *M* sodium phosphate (pH 7) or distilled water. The flow-rate was 0.48 ml/min in all separations, and the pressure did not exceed 10 atm. The void volume (V_0) and inner volume (V_i) were determined using Dextran T 2000 and glucose, with water as eluent. The total volume of the column was 10 ml, V_0 3.0 ml and V_i 5.35 ml.

RESULTS

Values of V_e and K_D for various bases, nucleosides and nucleotides, measured for a column (20 × 0.8 cm) packed with Spheron 100 using 0.1 *M* sodium phosphate (pH 7) as eluent, are given in Tables I and II. The partition coefficients, K_D, are defined as

$$K_D = \frac{V_e - V_0}{V_i}$$

where V_e is the elution volume of the compound tested, V_0 the void volume of the column and V_i the inner volume of the pores of the gel.

No significant differences in K_D values were observed using Spheron 100 and 1000, which differ in porosity (molecular weight exclusion limits for dextran standards 10^5 and 10^6 daltons, respectively). The HETP value for adenine was calculated as about 0.2 mm for both types of Spheron.

Table III gives the K_D values of some derivatives chromatographed on Spheron 100 using eluents of different ionic strength. Negligible swelling changes of the Spheron gels over a wide range of ionic strengths of the medium permitted the use of the same packed column for all experiments.

Examples of the chromatographic separations of some mixtures of bases, nucleosides and nucleotides on a column packed with Spheron 100 using elution with 0.1 *M* sodium phosphate buffer (pH 7) are given in Fig. 1–3. Fig. 1 shows the chro-

TABLE I

V_e AND K_D VALUES OF NUCLEIC ACID BASES AND THEIR ANALOGUES

Compound	V_e *(ml)*	K_D
Cytosine	8.9	1.10
Isocytosine	9.5	1.21
N-Acetylcytosine	11.6	1.61
Uracil	9.9	1.29
5-Aminouracil	8.6	1.05
5-Nitrouracil	15.2	2.28
5-Fluorouracil	11.8	1.64
5-Chlorouracil	17.8	2.77
5-Bromouracil	22.4	3.63
5-Iodouracil	34.9	5.96
5-Hydroxymethyluracil	8.6	1.05
Thymine	12.7	1.81
2-Hydroxypyrimidine	7.9	0.92
Barbituric acid	7.9	0.92
Hypoxanthine	11.3	1.55
Xanthine	12.9	1.85
Guanine	13.7	2.00
Adenine	21.7	3.50
2-Aminopurine	17.8	2.77
8-Azaadenine	16.6	2.54
4-Amino-[3,4-*d*]-pyrazolopyrimidine	37.9	6.52

TABLE II

V_e AND K_D VALUES OF NUCLEOSIDES AND NUCLEOTIDES

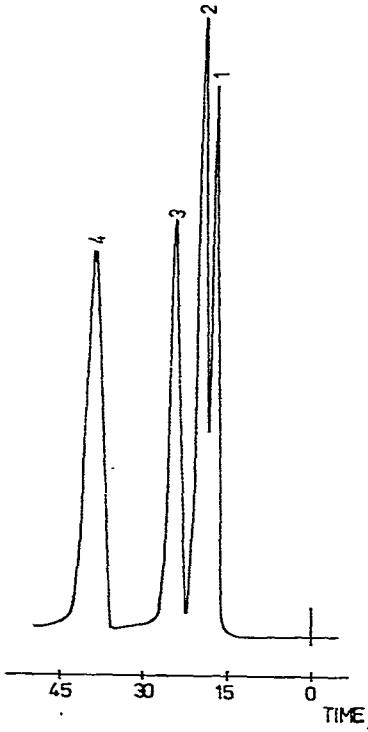
Compound	V_e *(ml)*	K_D
Cytidine	8.6	1.05
Uridine	9.7	1.25
Inosine	11.1	1.51
Guanosine	11.6	1.79
Adenosine	18.6	2.91
1-(β-D-arabinofuranosyl)uracil	10.0	1.31
2,2′-Anhydro-1-(β-D-arabinofuranosyl)uracil	8.5	1.03
2′-Deoxycytidine	9.5	1.21
2′-Deoxyuridine	11.3	1.84
5-Iodo-2′-deoxyuridine	37.0	6.35
Thymidine	13.7	2.00
2′-Deoxyguanosine	15.0	2.24
2′-Deoxyadenosine	25.6	4.22
Cytidine-5′-phosphate	6.5	0.65
Uridine-5′-phosphate	6.5	0.65
Guanosine-5′-phosphate	6.5	0.65
Adenosine-5′-phosphate	6.5	0.65
Thymidine-5′-phosphate	6.5	0.65
Adenosine-3′-phosphate	6.5	0.65
Adenosine-3′,5′-cyclic phosphate	11.8	1.64
Adenosine-5′-triphosphate	6.2	0.60

matogram of a mixture of four main ribonucleosides, Fig. 2 the separation of various adenine compounds and Fig. 3 the elution profile of a mixture of some 5-substituted derivatives of uracil. Fig. 4 illustrates the separation of uracil riboside and arabinoside in 0.1 *M* sodium phosphate buffer containing sodium chloride.

TABLE III

K_D VALUES OF SOME DERIVATIVES IN MEDIA OF DIFFERENT IONIC STRENGTHS

Compound	K_D			
	1 M NaCl in 0.1 M sodium phosphate (pH 7)	*0.1 M sodium phosphate (pH 7)*	*0.01 M sodium phosphate (pH 7)*	*Water*
Adenine	4.51	3.50	3.78	6.48
Adenosine	3.85	2.91	2.73	2.99
2′-Deoxyadenosine	5.69	4.22	3.78	4.11
Adenosine-3′,5′-cyclic phosphate	1.81	1.64	1.15	0.69
Uracil	1.35	1.29	1.38	1 41
5-Nitrouracil	1.81	2.28	1.38	0.69
5-Aminouracil	1.15	1.05	1.12	1.28
5-Hydroxymethyluracil	1.15	1.05	1.12	
Thymine	2.14	1.81	1.88	1.97
Uridine	1.35	1.25		
1-(β-D-arabinofuranosyl)uracil	1.55	1.31		



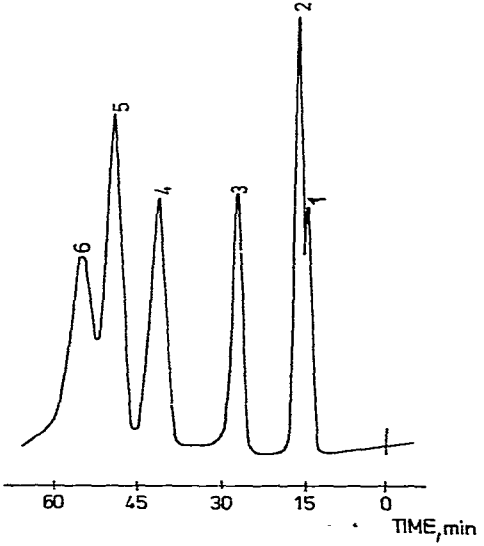


Fig. 1. Chromatogram of a mixture of four ribonucleosides on Spheron 100. Column, 20 × 0.8 cm; eluent, 0.1 *M* sodium phosphate (pH 7); flow-rate, 0.48 ml/min. Peaks: 1 = cytidine; 2 = uridine; 3 = guanosine; 4 = adenosine.

Fig. 2. Chromatogram of a mixture of adenine derivatives. Conditions as in Fig. 1. Peaks: 1 = adenosine-5′-triphosphate; 2 = adenosine-5′-phosphate; 3 = adenosine-3′,5′-cyclic phosphate; 4 = adenosine; 5 = adenine; 6 = 2′-deoxyadenosine.

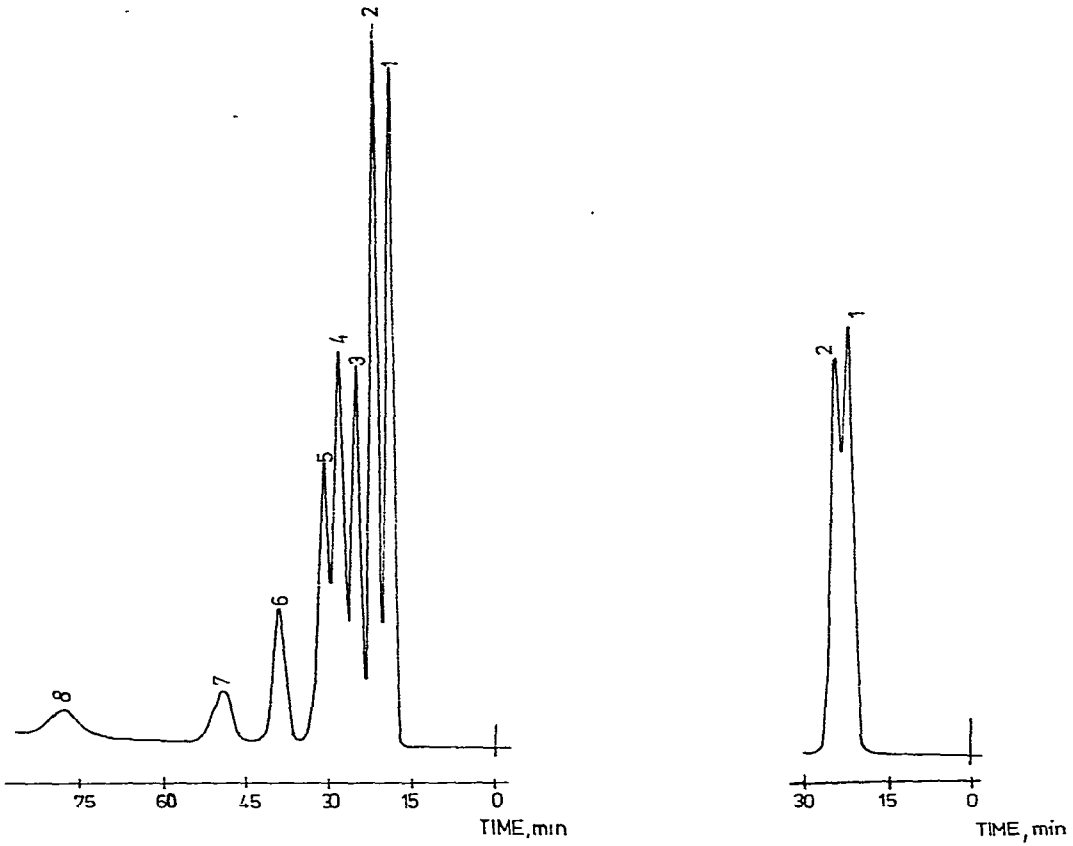


Fig. 3. Chromatogram of a mixture of 5-substituted uracil derivatives. Conditions as in Fig. 1. Peaks: 1 = 5-hydroxymethyluracil; 2 = uracil; 3 = 5-fluorouracil; 4 = thymine; 5 = 5-nitrouracil; 6 = 5-chlorouracil; 7 = 5-bromouracil; 8 = 5-iodouracil.

Fig. 4. Separation of uracil riboside and arabinoside. Eluent, 1 M NaCl in 0.1 M sodium phosphate (pH 7); other conditions as in Fig. 1. Peaks: 1 = uridine; 2 = 1-(β-D-arabinofuranosyl)uracil.

DISCUSSION

Partition coefficients reflect the differences in energy of isolated systems, solvated molecules of solute–solvated gel surface, and that of interacting systems, solute molecules–gel surface–solvent. This difference in energy is influenced by the electronic structure of the solute and hence by its substitution. The effect of substitution on the chromatographic behaviour of purine and pyrimidine derivatives was similar for hydroxyethyl methacrylate gels and for dextran or polyacrylamide gels[4,6]. An interesting exception was the chromatographic behaviour of deoxyribonucleosides and corresponding bases: bases were eluted from Spheron by 0.1 *M* sodium phosphate before deoxynucleosides; the opposite order was observed for Sephadex[4,6]. In the latter instance, however, elution was performed with more dilute buffers. A study of the chromatographic behaviour of the pair adenine–2′-deoxyadenosine showed that their elution order can vary with the ionic strength of medium. As indicated in Table III, in 0.1 *M* sodium phosphate buffer, or in this buffer plus sodium chloride, deoxynucleoside was

eluted after the base, in 0.01 *M* buffer both compounds were eluted together and in water deoxyadenosine preceded adenine. The K_D value of 2'-deoxyadenosine decreased with a decrease in ionic strength up to 0.01 *M* buffer, whereas for adenine the K_D value was at a minimum in 0.1 *M* buffer.

An enhanced ionic strength of the medium quenches electrostatic effects and favours interactions between the solute and the gel surface realized by short-distance forces. Hence, in buffers of higher ionic strength, most of the derivatives tested showed increased retentions. However, on comparison of the system 0.1 *M* sodium phosphate–1 *M* sodium chloride with 0.1 *M* sodium phosphate, not only the difference in ionic strength, but also the change of the type of main electrolyte in solution should be considered. A lyotropic effect, due to this change, may cause an anomalous behaviour of 5-nitrouracil (the K_D value of this compound showed a maximum in 0.1 *M* sodium phosphate, while addition of sodium chloride decreased the retention).

In very dilute buffers or in water, the chromatographic behaviour of particular compounds was also influenced by ionic interactions, due to presence of carboxylic groups in the gel (0.013 mequiv. titritable COOH groups per gram). In these media, derivatives with basic groups are more retained as a consequence of ion exchange. On the other hand, the ion exclusion of acidic compounds (*e.g.*, adenosine-3',5'-cyclic phosphate or 5-nitrouracil) causes a decrease in their K_D values to below 1.

The differences in the K_D values of particular compounds are sufficient to permit the fractionation of various mixtures of important bases and nucleosides. Some of these mixtures are difficult to resolve by ion exchange. Fractionation of nucleic acid components can be achieved without gradient elution and in neutral media. Nucleoside mono-, di- and triphosphates can be separated from corresponding cyclic phosphates, nucleosides and bases (Fig. 2). Even similar bases or nucleosides were separated, e.g., 5-substituted uracil derivatives (Fig. 3) or uracil riboside and arabinoside (Fig. 4). Fractionation of nucleotides was not achieved on unmodified Spheron; for this purpose, however, ion-exchange derivatives of Spheron could be advantageous[7].

The conditions used for the fractionations shown in Fig. 1–4 were not optimized. Nevertheless, owing to the macroporous character of Spheron gels and hence the rapid diffusion into and out of the gel particles, a high efficiency of separation was achieved (the HETP value for adenine was 0.2 mm). This separation efficiency is much higher than that in dextran gel chromatography[6], and is comparable to the efficiency of ion-exchange and ion-exclusion chromatography of nucleic acid components[8]. In particular instances, even better resolutions could be obtained by changging the ionic strength, pH and composition of the eluting buffer, the operating temperature, flow-rate, column dimensions and the particle size of the gel beads.

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