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LIQUID COLUMN CHROMATOGRAPHY OF NUCLEOSIDE MONOPHOSPHATES ON DEAE-SPHERON 1000

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SUMMARY

The ion exchange capacity, radiation stability and particle distribution have been estimated for two fractions of the weak anion exchanger DEAE-Spheron 1000. Capacity factors are reported for eight nucleotides measured under various conditions in citrate and formate buffers. This type of sorbent may be used for the separation of deoxy- or ribonucleotides under mild conditions in ammonium formate buffer. No interferences from other substances which are usually found in samples of biological origin have been observed.

INTRODUCTION

Supports which have been used for the separation of nucleotides are either polystyrene-based strong anion exchangers such as Aminex A-27, A-28[1–3], chemically bonded strong anion exchangers on silica microparticles[4,5] or weak anion exchangers with a cellulose or dextran matrix such as DEAE-Sephadex[6,7]. To these well established column materials may be added[8,9] octadecyl-bonded silica.

However, all separation methods using these supports possess certain disadvantages. Thus the polystyrene-based anion exchangers, most commonly used, are chemically and thermally stable, but the aromatic rings within the matrix cause strong adsorption of the aromatic parts of nucleotides. This may be suppressed by increasing the temperature (usually to 70°), but this increases the rate of decomposition (hydrolysis) of nucleotides, especially in acidic buffers. On the other hand, the use of basic pH values may cause overlap of the elution volumes of nucleosides and nucleotides[3] and a two-step separation procedure may be needed. The silica-bonded ion exchangers increase the separation speed and may be used at room temperature, but their exchange capacity is usually low and some organic compounds, which may occur frequently in biological samples, may drastically decrease their efficiency[5]. The dextran-based ion exchangers are produced as relatively coarse particles and are easily compressible, therefore are not suitable for high-performance liquid chromatography. Finally, separation of nucleotides by reversed-phase chromatography on octadecyl bonded to silica has been described only recently[8,9] and its scope seems to be limited.

DEAE-Spheron is a weak anion exchanger produced by substitution of hydroxymethyl methacrylate–ethylenedimethacrylate copolymer beads[10] and has been used for the separation of a broad range of biologically important substances, *e.g.*, sugars[11] and mono-, di- and triphosphates of adenosine[12]. It is more hydrophilic than polystyrene-based anion exchangers, its rigidity in the swollen state is satisfactory and its capacity is higher than that of silica-based ion exchangers. Therefore it seemed worthwhile to try it for the separation of naturally occurring 5′-nucleotides.

EXPERIMENTAL

Apparatus

Glass columns of various lengths and inner diameter of 3.5 mm were used[13]. The eluents were stored in glass containers, degassed by heating to 70° and transported by pump MC 706.1 equipped with pumping head K or L (Mikrotechna, Prague, Czechoslovakia). A Bourdon-type manometer was used for pressure monitoring (Chirana, Prague, Czechoslovakia). A short precolumn (20 mm × 4 mm I.D.) filled with DEAE-Spheron 1000 was used for removal of particulate matter or impurities from the eluent. The eluent was monitored by a Uvicord III (LKB, Stockholm, Sweden) and by a conductivity detector described previously[14]. Signals were recorded by a four-channel Type 175 recorder (Kutesz, Budapest, Hungary).

Reagents

Adenosine 5′-monophosphate, sodium salt (AMP) (type II), 2′-deoxyguanosine 5′-monophosphate (dGMP), deoxycytidine 5′-monophosphate (dCMP), 2′-deoxyadenosine 5′-monophosphate (dAMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), uridine 5′-monophosphate (UMP) and thymidine 5′-monophosphate (TMP) were purchased from Sigma (Saint Louis, Mo., U.S.A.); all other reagents of p.a. quality were from Lachema (Brno, Czechoslovakia).

Sorbent

The sorbent DEAE-Spheron 1000 was a Lachema product and two fractions were available to us, having nominal particle diameters of 25–40 μm and of < 25 μm, respectively.

Particle size determination

Sorbent particles swollen in distilled water were photographed using an Amplival optical microscope (Carl Zeiss Jena, Jena, G.D.R.) with × 160 magnification optics, Exacta camera and a high-resolution ORWO NP 15 film. At the same setting, several frames were exposed to depict more than 500 particles, and a glass scale divided into 10-μm sections. From prints made under identical conditions, the diameters of 500 randomly selected particles were measured with a precision of *ca.* 0.1 mm (3–5 mm being the diameter of particles on the print). The measurements were statistically evaluated using a simple program written in BASIC (Varian 620 L computer). The diameter of the majority of particles ($> 96\%$) lies between 14 and 40 μm for the 25–40-μm fraction and between 8 and 29 μm for the <25-μm fraction. This distribution seems suitable for most preparative applications, but for some demanding separations the spread of particle sizes should be narrower. Statistical characteristics of both fractions are summarized in Table I.

TABLE I

DISTRIBUTION OF PARTICLE SIZES

Parameter	*Fraction 1*	*Fraction 2*
Nominal diameter (μm)	25–40	<25
Number average (μm), M_n	25.87	17.42
Weight average (μm), M_w	27.45	18.78
Standard deviation (μm), σ_n	6.41	5.02
Skew	0.69	0.18
Excess	0.46	0.01

Packing of columns

Typically, *ca.* 3 g of Spheron were stirred with ca. 50 ml of 1 *M* buffer at an appropriate pH and left to stand for *ca.* 1 h; then the supernatant was decanted and the whole procedure repeated five or six times. Finally, the wet sorbent was suspended in a concentrated solution of buffer and the slurry poured into a reservoir (100 mm × 18 mm diameter), with a column of the same solution attached to its lower end. Buffer solution (*ca.* 1 *M*) was fed into the reservoir at a pumping speed of *ca.* 26 ml/h. When the column was completely filled with sorbent, the reservoir was disconnected, the injector-head mounted on the column and the column washed with buffer of desired molarity and pH. This procedure guarantees attainment of high quality packing and is superior to all others we have tried (high-pressure, high-viscosity and low-density slurry packing).

RESULTS AND DISCUSSION

Measurement of interstitial volume

The determination of interstitial volume is important for the estimation of capacity factors. However, all of the nucleic bases, nucleosides and other compounds that we tried were to some degree retained on the column. The interstitial volume (*i.e.*, the total volume within the column accessible to the eluent) was best determined by conductimetric measurements of the slight fluctuations of buffer concentration in the effluent at the exit from the column. The column (250 × 3.5 mm), filled with DEAE-Spheron 1000 (25–40 μm), was operated at 50°. As elunt, 0.07 *M* ammonium formate buffer (pH 4.5) was used. When 50 μl of 0.5 *M* ammonium formate were injected, a conductivity peak (conductivity increase) at an elution volume of 1.58 ml was found. Under identical conditions, but 50 μl of water injected, an interstitial volume of 1.49 ml was found (eluent conductivity decrease peak). The larger volume found with the increased concentration of eluent, (*i.e.*, an increase of conductivity) is due to marked asymmetry of that peak, however, the difference in retention volumes is very small (*ca.* 6%). Changes in temperature (20–70°) pH (3–5), injected volume (10–50 μl) and concentration of injected formate (0.5–2 *M*) had no influence on the interstitial volume. An increase of eluent concentration (from 0.07 to 0.4 *M*) causes the interstitial volume to increase by 7%. A decrease in the mean particle size of the packing from 26 to 27 μm resulted in the interstitial volume increasing from 66 to 73% of the void volume of the column. When the length of the column packing was increased from 227 mm to 977 mm, there was no change in the relative interstitial volume.

Capacity of DEAE-Spheron 1000

Nominal ion exchange capacity values, supplied by the producer, were 1.5 ± 0.25 mequiv./g (dry sorbent) for the 25–40 μm fraction and 1.68 mequiv./g for the <25 μm fraction[15]. The ion exchange capacity of sorbent was determined by titration with perchloric acid. It is well known that the perchlorate ion has a high affinity for most anion exchangers. The dry sorbent was weighed and *ca.* 1 g was transferred to a beaker, rinsed five times with 30 ml 1 *M* NaOH, then (on a frit funnel) washed with 0.8 l of freshly boiled distilled water. It was then suspended in 20 ml water and titrated with 0.1 *M* $HClO_4$. The single additions of acid solution were *ca.* 1 ml; after every addition the solution was stirred until a steady pH was reached (*ca.* 15 min). The capacity so determined was 1.51 mequiv./g for the coarser fraction, in good agreement with the manufacturer's data.

The maximum amount of nucleotide which may be retained under typical conditions on the column packing was estimated from a break-through experiment. The column (61 × 3.5 mm) was equilibrated with 0.07 *M* ammonium formate and then a solution of AMP in water (1.62 mg/ml) was pumped through the column. As may be seen from Fig. 1, the inflection point in the concentration *vs.* volume curve corresponds to 13.2 mg AMP retained on the column, *i.e.*, to 0.25 mmol/g of dry sorbent. If the concentration of AMP solution was increased to 6.48 mg/ml, the inflection point corresponded to 0.67 mequiv./g of dry sorbent. Thus a fourfold increase of concentration corresponds to only a 2.7-fold increase of sorbed AMP. Obviously, if the sorption isotherm is no longer linear, even at relatively high concentration only a fraction of exchange sites is used.

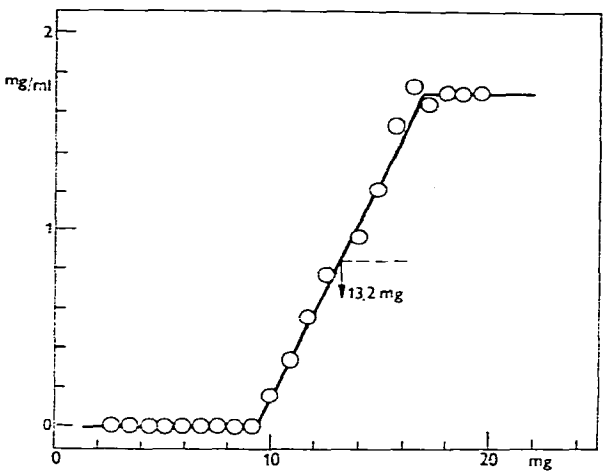

Fig. 1. Determination of capacity of DEAE-Spheron 1000 for AMP. Sorbent fraction: <25 μm. Eluent: solution of AMP in water, 1.62 mg/ml. Interestitial volume: 0.47 ml.

Citrate buffer

All measurements with citrate buffer were performed at 50° and pH 3.5. The concentration of buffer is given here as the molarity of sodium citrate; the citric acid used for the adjustment of acidity was not taken into account. So the nominal concentration corresponds approximately to the citrate-ion concentration. All com-

putations were performed using programs GRABAC and GRAPUR in BASIC (Varian 620 L computer). Approximately linear plots of log K_D *vs.* log c were obtained, with a slope of *ca.* −0.5 (Fig. 2). For monophosphates of ribonucleosides the critical pair is AMP–UMP, for the monophosphates of deoxyribonucleosides it is dAMP and dTMP. The separation of deoxy- and riboso-pairs derived from the same base is also poor. The influence of pH is more marked for the nucleotides derived from amino-substituted bases (CMP, dCMP, AMP, dAMP). Thus, on going from pH 3.5 to pH 4.5, the elution sequence of AMP and UMP (respectively dAMP–dUMP) is reversed. At the same time, the resolution of the deoxy- and riboso-pair improves; however, with a relatively short column it is still rather poor.

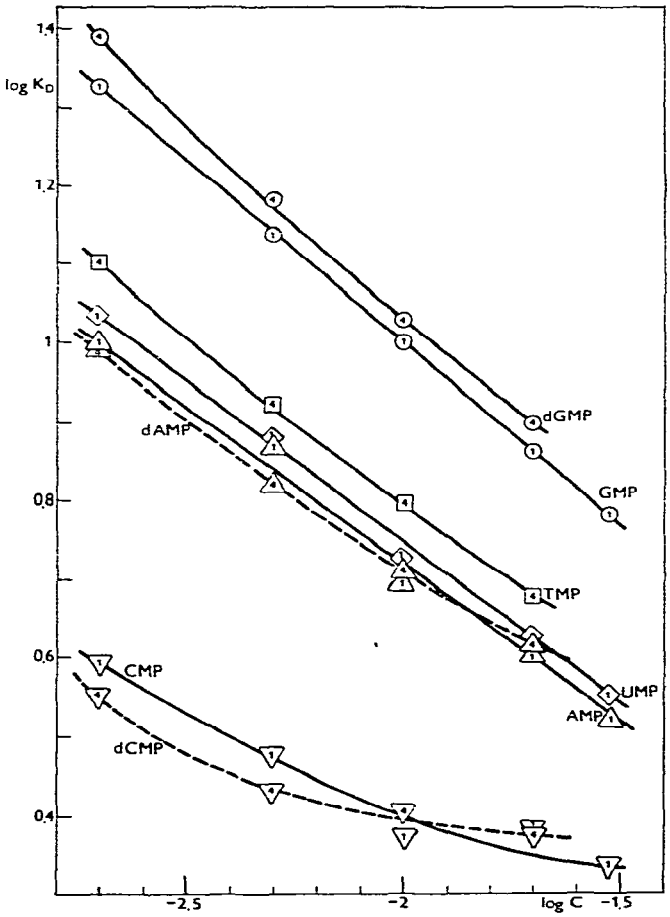

Fig. 2. Plot of capacity factors *versus* buffer concentration (logarithmic scales). Buffer: sodium citrate, pH 3.5. Column (250 × 3.5 mm) temperature: 50°. Pumping speed: 25 ml/h. Sorbent fraction: 25–40 μm.

Formate buffer

As with citrate buffer, the concentration of the formate buffer indicated in the graphs and tables corresponds to the molarity of ammonium formate; the pH was adjusted by addition of formic acid (pH<6) or ammonia.

It is interesting to compare the results obtained with citrate and formate buffer at the same pH (Figs. 2 and 3). Formate ions have a much weaker affinity for the sorbent than citrate ions and in order to attain the same elution volume, the concentration of formate must be about 10 times greater. The plots of log K_D *vs.* log c are approximately linear, and the slope approaches the theoretical value of 1.0. The elution sequence in both buffers at pH 3.5 is the same for all eight nucleotides.

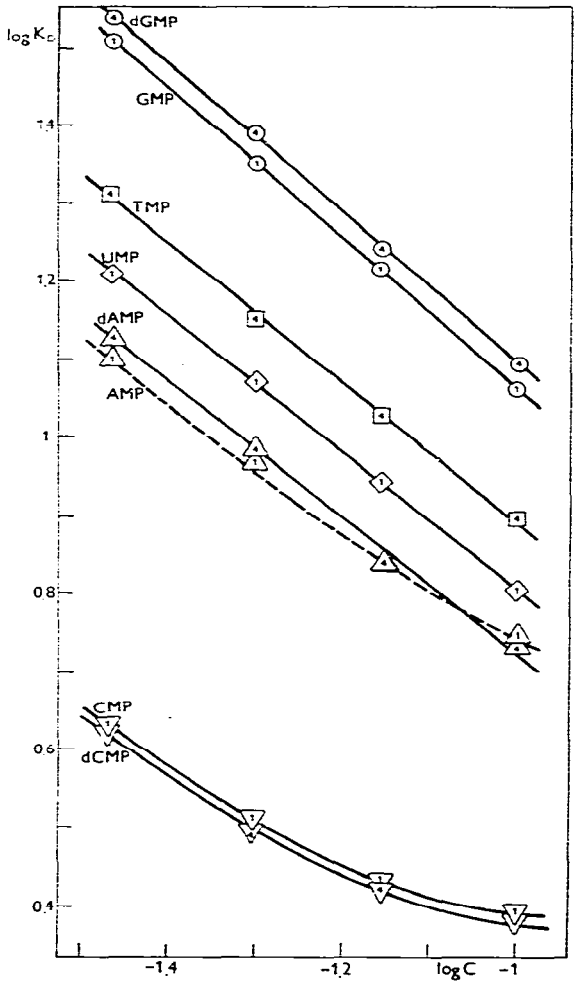

Fig. 3. Influence of concentration on capacity factors of nucleoside monophosphates. Eluent: ammonium formate buffer, pH 3.5. For other parameters see Fig. 2.

The influence of pH on capacity factor (in 0.07 M ammonium formate) is shown in Fig. 4. It can be seen that the optimum pH value for the separation of four ribonucleotides or deoxyribonucleotides is around pH 3 or pH 4.5. If all eight nucleotides are separated, the optimum pH value is 4.7. A change of elution sequence occurs at pH 3.7–4.0. At pH 7 the elution character is drastically changed: the differences between

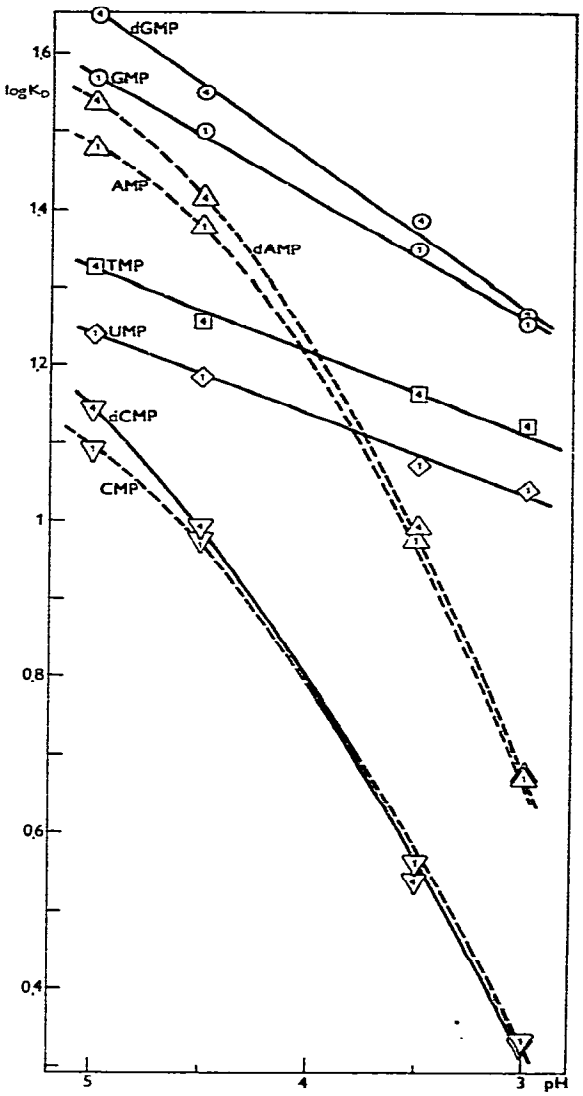

Fig. 4. Influence of pH on capacity factors of nucleoside monophosphates. Eluent: ammonium formate buffer, 0.07 *M*. For other parameters see Fig. 2.

purine or pyrimidine nucleotides are negligibly small, and the separation of AMP and GMP is practically impossible.

When the acidity of both the formate and citrate buffers is decreased, the peak widths increase. At higher eluent concentration and constant pH the peak width decreases.

Column temerature and flow-rate

The influence of temperature on the separation of nucleotides on DEAE-Spheron 1000 was followed quantitatively for the CMP–UMP pair. Results were collected for several consecutive days at temperatures of 30° and 50°. Using the

Student's criterion, it could be estimated (at the 99.9% confidence level) that the temperature markedly influences the resolution of this pair (see Table II). It is of interest that the elution volume of UMP decreases with increasing temperature, but that for CMP the retention is stronger at higher temperature. The column efficiency for UMP decreases with increasing temperature (99.9% confidence level), whereas the reverse is true for CMP. Finally, the higher resolution of this pair at lower temperature is mainly due to the change of retention volumes.

TABLE II

INFLUENCE OF TEMPERATURE ON SEPARATION OF CMP AND UMP

N = Number of theoretical plates, W = peak width, V_R = elution volume, R = resolution. Column, 250 × 3.5 mm; eluent, 0.07 M ammonium formate (pH 4.5); pumping speed, 9.8 ml/h. Sorbent: DEAE-Spheron 1000, 25–40 μm.

Compound	*T (°C)*	*V_R (ml)*	*W (ml)*	*N*	*R*	*No. of experiments*
CMP	30	5.185 ± 0.039	0.795 ± 0.014	679 ± 28	3.50	11
UMP	30	8.899 ± 0.091	1.329 ± 0.019	717 ± 18		
CMP	50	5.588 ± 0.020	0.799 ± 0.017	784 ± 32	2.64	8
UMP	50	8.370 ± 0.027	1.311 ± 0.026	652 ± 25		

If the linear speed of the eluent is decreased from 0.26 cm/sec to 0.014 cm/sec, the HETP decreases to about one fourth and resolution is increased *ca.* 1.8 times.

Radiation stability of DEAE-Spheron 1000

A short column filled with sorbent and buffer was subjected to irradiation by high-energy photons in a Gamacell radiation source (^{60}Co). The results are summarized in Table III. Even the highest absorbed dose (161 kiloGrey) did not markedly change the separation efficiency of this column. So, also in this respect the DEAE-Spheron 1000 compares favourably wtih polystyrene-based ion exchangers.

TABLE III

IRRADIATION OF CHROMATOGRAPHIC COLUNN

Column, 3.5 × 95 mm; pumping speed, 13.2 ml/h; DEAE-Spheron 1000, ~ 25 μm.

Dose absorbed (Gy)	*CMP*			*AMP*		
	K_D	*N*	*R*	K_D	*N*	*R*
0	1.74	290	—	9.59	387	5.64
$1.44 \cdot 10^4$	1.61	239	—	9.19	370	5.40
$4.032 \cdot 10^4$	1.65	234	—	9.55	376	5.57
$16.128 \cdot 10^4$	1.65	281	—	9.30	351	5.41

CONCLUSION

DEAE-Spheron 1000 may be used both for analytical and preparative separations of nucleotides from biological samples. The separation may be conducted under mild conditions (room temperature, pH 4.7). No interference from

substances commonly occurring in biological extracts (*e.g.*, Tris buffer) has been observed. The group separation of nucleosides and bases from the first eluting nucleotide, CMP, is good and therefore the one-step separation is satisfactory (see Fig. 5). The rigidity of the swollen gel seems to be higher than that of polystyrene-based ion-exchangers so that pressure gradients up to 100 kPa/cm may be used safely. The exchange capacity is similar to that of polystyrene-based ion exchangers and this may be used even for preparative separations. The peak widths under comparable conditions are much smaller on DEAE-Spheron gel than on polystyrene-based anion exchangers. Work is in progress in this laboratory to demonstrate the application of this gel for the separation of very complicated mixtures of nucleotides and similar substances.

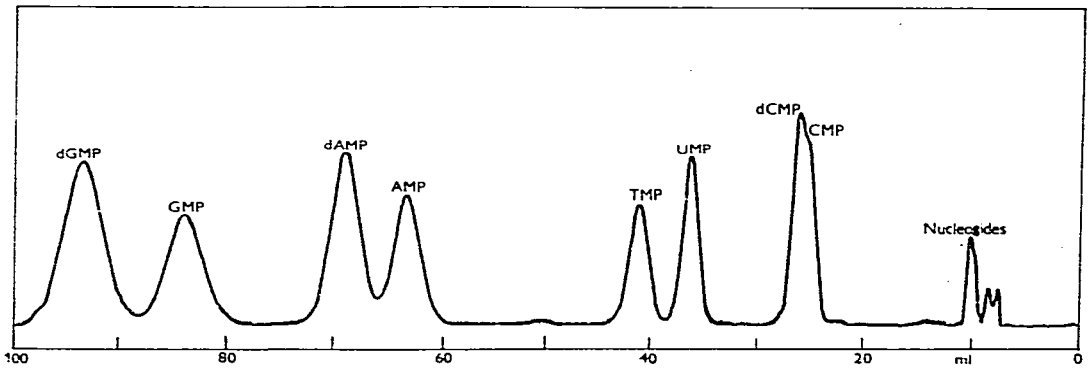

Fig. 5. Elution pattern of synthetic mixture of nucleosides and nucleoside monophosphates. Eluent: 0.07 *M* ammonium formate, pH 4.7. Column (980 × 3.5 mm) temperature, 50°; pressure, 1.2 MPa, Pumping speed: 26 ml/h. Sorbent: DEAE-Spheron 1000; fraction, 25–40 μm.

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