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Efficient separation of natural ribonucleotides by low-pressure anion-exchange chromatography

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ABSTRACT

Chromatographic conditions for the separation of twelve purine and pyrimidine ribonucleotides (mono-, di- and triphosphates) by ion-exchange chromatography were investigated. Two types of anion exchangers with tertiary or quaternary ammonium functionalities (DEAE and QAE types) were compared. Parameters examined included pH of the mobile phase, elution buffer composition and flow-rate. Excellent resolution of all compounds in 50 min was achieved with a strongly basic anion exchanger in the formate form using a linear gradient of ammonium formate with constant formic acid concentration in the mobile phase. The proposed method utilizes (1) a strongly basic anion exchanger based on a macroporous hydrophilic organic polymer, (2) a volatile elution buffer system which permits one-run purification of nucleotides (no further column operation is required, *e.g.*, desalting) and (3) low-pressure liquid chromatographic equipment with simple column preparation. The method was applied to the purification of [U-^{14}C]adenosine-5′-diphosphate and other radiolabelled nucleotides and their analogues in order to obtain highly radiochemically pure compounds.

INTRODUCTION

Forty years ago, a strong anion exchanger (based on styrene–divinylbenzene copolymer, Dowex type) was applied for the first time for the separation of nucleotides [1], and subsequently the same material has frequently been utilized for this purpose [2–4]. DEAE-Sephadex [5], PEI-cellulose [6], acriflavine gel [7] and, in particular cases, cation exchangers [8,9] were also applied for separations of nucleotide mixtures.

The introduction of microparticular materials as efficient packing sorbents [10–12] allowed the development of high-performance liquid chromatography (HPLC). Several workers have reviewed methods of nucleotide analysis using HPLC [13–15]. This area includes two methodologies: anion-exchange chromatography [16–21] and reversed-phase chromatography on octadecyl-bonded silica gel [22–24]. The latter is often used in a mode of ion-pair chromatography employing tetra-*n*-butylammonium [25–27], trimethyl- or triethylammonium [28] and *n*-heptyltriethylammonium [29] cations as counter ions. Modern high-performance anion-exchange chromatography mostly utilizes quaternary ammonium functionalities and phosphate- or chloride-based buffers. To increase the column efficiency and/or selectivity of separation, water-miscible organic modifiers [17,30–32] have been introduced.

Our previous experience with nucleoside and nucleotide separations using a strongly basic anion exchanger of the benzyltrimethylammonium type [33] based on a rigid macroporous hydrophilic copolymer of 2-hydroxyethyl methacrylate and ethylene glycol dimethacrylate [34–36] (Separon HEMA series) prompted us to use

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in further studies medium-basic (diethylaminoethyl type) and strongly basic (triethylammoniumethyl type) exchangers derived from the same matrix. These commercially available exchangers exhibit very good mechanical properties, excellent chemical resistance at extreme pH values and negligible volume changes caused by variations in ionic strength and pH of the mobile phase.

In this work, we studied the chromatographic behaviour of twelve ribonucleotides with respect to the pH and composition of the mobile phase, flow-rate and type of anion exchanger used. The influence of these parameters on the elution order of the compounds is discussed. We found conditions for the baseline separation of all twelve components using a strongly basic anion exchanger (QAE type) in the formate form. We applied this chromatographic system to the purification of some ^{3}H- and ^{14}C-labelled nucleotides and their analogues intended for metabolic studies. In all instances we achieved highly efficient separations and obtained compounds with radiochemical purities of more than 98%.

Although this chromatographic work is directed towards the area of the micropreparation of nucleotides from mixtures after chemical or enzymatic synthesis at micromolar levels, the method can also be used for the analytical separation of nucleotides in biological materials. Further studies concerning especially the influence of temperature on the nucleotide separation and optimization of the anion-exchange packing procedure are in progress.

EXPERIMENTAL

Separon HEMA-1000 Q (QAE type, particle size 10 μm, 0.60 mequiv./g) and Separon HEMA-1000 DEAE (DEAE type, particle size 15 μm, 1.22 mequiv./g) (the molecular mass exclusion limit of the basic matrix is 10^6, determined for dextrans), were obtained from Laboratorní přístroje (Prague, Czech Republic). 5′-Monophosphates of adenosine (AMP), cytidine (CMP), guanosine (GMP), uridine (UMP) and cytidine 5′-triphosphate (CTP) were purchased from Sigma (St. Louis, MO, USA). 5′-Diphosphates of adenosine (ADP), cytidine (CDP), guanosine (GDP) and uridine (UDP) and 5′-triphosphates of adenosine (ATP), guanosine (GTP) and uridine (UTP) were obtained from Boehringer (Mannheim, Germany).

The liquid chromatograph consisted of an LCP 3001 high-pressure, pulse-free pump with a GP 3 gradient programmer (low-pressure side gradient formation), a UV detector operating at 254 nm, a TZ 4620 line recorder and a CI 100 computing integrator (all from Laboratorní přístroje). A sample loop injector was purchased from Knauer (Bad Homburg, Germany). A glass column (150 mm × 8 mm I.D.) including adjustable endpieces was made in the Institute mechanical workshop.

Preparation of the anion-exchanger column

Ion exchangers were converted into the chloride form by gradual rinsing with sodium hydroxide (1 mol/l; 50 ml per gram of the resin), water, dilute hydrochloric acid (1 mol/l; 100 ml per gram of the resin) and again with water to neutral pH of the filtrate. Further decantation of the resin in acetone (100 ml/g; three times) provided dust-free material which was dried *in vacuo* at room temperature for 15 h. A slurry of the exchanger in water (20%, w/v) was transferred into the glass column at a flow-rate of 3 ml/min. After bed settling (bed height *ca.* 130 mm) the column was closed with an adjustable end-piece and the bed was then compressed by hand to the final height of 120 mm. The column was washed either with potassium chloride solution (1 mol/l, 50 ml) or with ammonium formate solution (2 mol/l, 100 ml) at a flow-rate of 3 ml/min and finally equilibrated with the appropriate starting buffer.

Chromatography

Chromatography was usually performed for 60 min at a flow-rate of 1 ml/min. The pressure never exceeded 10 bar. Elution of nucleotides from the resin in the chloride form was achieved using a linear gradient of potassium chloride from 0.05 to 0.35 mol/l at various pH values of the leading buffer (potassium phosphate, 0.05 mol/l). The appropriate pH was adjusted by

addition of dilute phosphoric acid or potassium hydroxide solution.

In separations using a strong anion exchanger in the formate form, a linear gradient of ammonium formate from 0 to 0.6 mol/l at various formic acid concentrations (ranging from 0 to 0.15 mol/l) was used.

A representative mixture of nucleotides was injected into the column in microgram amounts (10 μg of each per 100 μl of the sample).

Determination of radiochemical purity

The samples (2 kBq) were analysed by ion-pair HPLC using a 250 × 4.6 mm I.D. Separon RPS SGX (7 μm) column (Tessek, Prague, Czech Republic). Elution buffer A contained 0.05 mol/l $(NH_4)_2HPO_4$ and 0.003 mol/l tetrabutylammonium hydrogensulphate (pH 6.8); buffer B had the same composition but contained 20% (v/v) acetonitrile. The separation was carried out for 30 min with a linear gradient from A–B (95:5) to A–B (40:60) and with a flow-rate of 1 ml/min. The radioactivity of 0.5-ml fractions was measured using a Beckman LS-6000A scintillation counter following the addition of 5 ml of Aquasafe 300 scintillation cocktail (Zinsser Analytic, UK).

RESULTS AND DISCUSSION

Effect of pH and buffer composition

The pH of the mobile phase is one of the most important factors for the resolution of species by ion-exchange chromatography. In this work, the effect of pH in the range 3–9 on the retention time (expressed as the capacity factor, k') was studied. Generally, both exchangers showed the expected separation patterns (Fig. 1a and b).

At low pH, the retention was decreased owing to the mutual weaker ionic interactions between nucleotides and the exchanger. This is caused both by suppression of phosphate group dissocia-

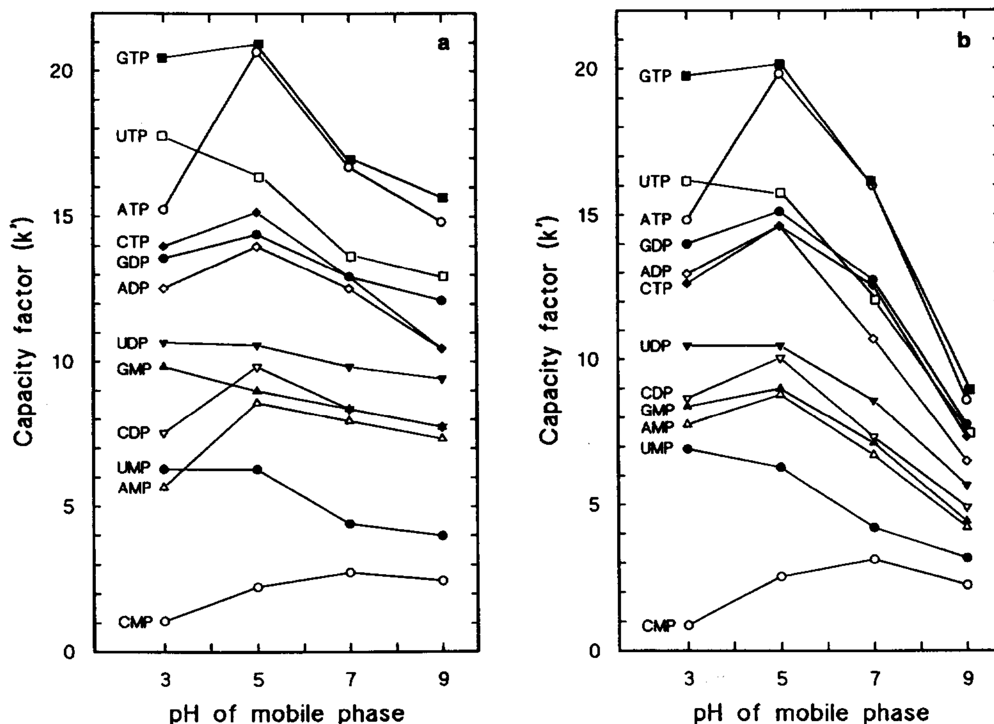

Fig. 1. Dependence of the capacity factor (k') of particular ribonucleotides on the pH of the mobile phase. Separations were performed on (a) Separon HEMA-1000 Q and (b) Separon HEMA-1000 DEAE with a 60-min linear gradient of KCl from 0.05 to 0.35 mol/l at a flow-rate 1 ml/min. The mobile phase contained 0.05 mol/l potassium phosphate adjusted to a given pH.

tion and by protonation of the amino groups of cytosine, adenine and guanine. In our experiments, adenine nucleotides showed the largest and uracil nucleotides the smallest shortening of retention. Although the retention maximum of most of the compounds started around pH 5, such conditions interfere with the desired separation, especially of purine nucleotides, using either exchanger.

As the pH increases to the basic region, the retention of nucleotides decreases and different behaviours of the two exchangers are observed. The shortening of retention is greater for the medium-basic anion exchanger because partially deprotonated diethylaminoethyl groups of the resin do not permit such strong interactions with phosphate groups of the nucleotides.

The desired separation of nucleotides using the QAE-type exchanger in the chloride form was achieved at pH 3, where only the CTP–GDP pair was not separated (Fig. 2).

In subsequent studies the QAE type of exchanger in the formate form was chosen. It was observed that not only the ionic strength but also the concentration of formic acid in the mobile phase substantially affect the quality of separation. We kept the gradient slope and concentration range of ammonium formate constant and in each run only the molarity of formic acid was changed. It was found that formic acid at concentrations up to 0.09 mol/l significantly affected capacity factors of the nucleotides (Fig. 3). Above this concentration (up to 0.15 mol/l), the capacity factors were found to be independent of formic acid concentration (data not shown). The best resolution of all twelve ribonucleotides was achieved at 0.06 mol/l formic acid. We also studied this separation system using a basic pH of the mobile phase. In this instance formic acid was replaced with ammonia solution. However, the quality of the separation decreased very significantly under these conditions.

An attempt to increase the efficiency of nucleotide separation using organic modifiers in the mobile phase [methanol and acetonitrile up to 50% (v/v) were used] failed completely. Contrary to expectation, we observed a significant

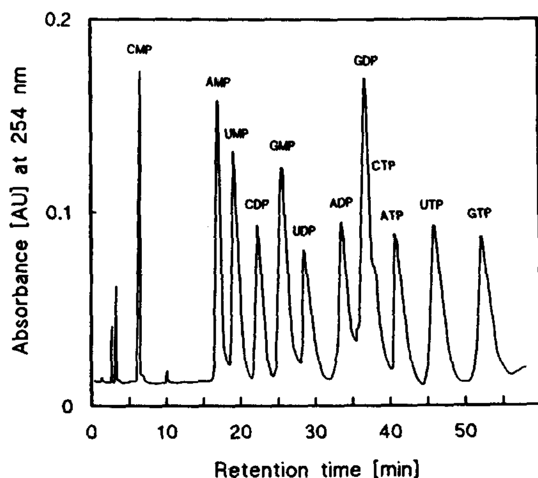

Fig. 2. Chromatography of a mixture of twelve ribonucleotides on Separon HEMA-1000 Q under the conditions described in Fig. 1. The pH of the mobile phase was adjusted to 3.0.

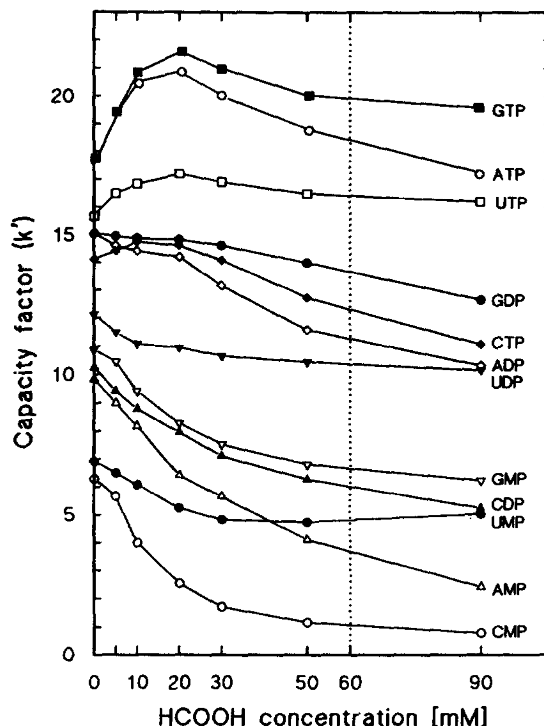

Fig. 3. Dependence of the capacity factor (k') of individual nucleotides on the concentration of formic acid in the mobile phase. Separations were performed on Separon HEMA-1000 Q with a 60-min linear gradient of ammonium formate from 0 to 0.60 mol/l at a flow-rate 1 ml/min. The mobile phase contained an appropriate concentration of formic acid.

decrease in the efficiency of separation (data not shown).

Effect of flow-rate

Under the most favourable chromatographic conditions using the exchanger in the formate form, we studied the influence of flow-rate on the quality of resolution (expressed as the resolution factor R_s) in the range 0.5–1.5 ml/min. The separation of four pairs of nucleotides was substantially affected by this parameter (Fig. 4). We found the usual dependence for the well separated pairs CDP–GMP, UDP–ADP and CTP–GDP up to 1.2 ml/min. Higher flow-rates decreased only the resolution of CDP–GMP.

A completely different behaviour was found for the ADP–CTP pair. We did not observe any significant resolution at flow-rates up to 1.0 ml/min, but a dramatic change occurred at 1.2 ml/min, where a baseline separation was obtained.

The optimum resolution of all twelve nucleotides was achieved at a flow-rate of 1.2 ml/min (linear speed 2.4 cm/min). Fig. 5 shows the chromatogram for this run and Table I gives the retention times (t_R), capacity factors (k'), resolution factors (R_s), number of theoretical plates (N), heights equivalent to a theoretical plate (HETP) and asymmetry factors (A_s).

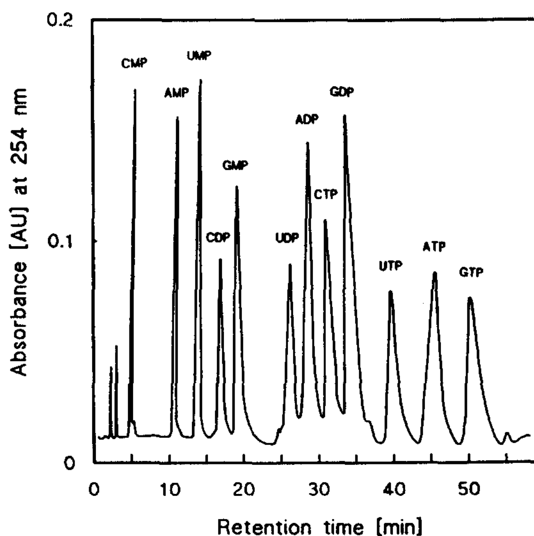

Fig. 5. Chromatography of a mixture of twelve ribonucleotides on Separon HEMA-1000 Q under the conditions given in Fig. 4. The flow-rate of the mobile phase was 1.2 ml/min.

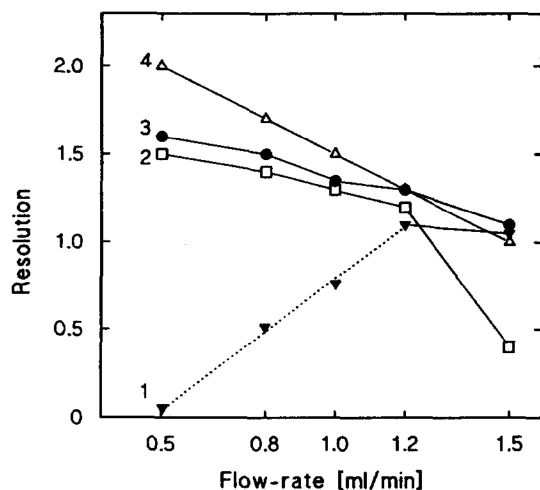

Fig. 4. Dependence of the resolution factor (R_s) of four neighbouring pairs of nucleotides on the flow-rate of the mobile phase. The separations were performed on Separon HEMA-1000 Q using a 60-min linear gradient of ammonium formate from 0 to 0.60 mol/l. The mobile phase contained 0.06 mol/l formic acid. Pairs of nucleotides: 1 = ADP–CTP; 2 = CDP–GMP; 3 = CTP–GDP; 4 = UDP–ADP.

Use of the system for purification of radiolabelled nucleotides

Chromatography on the strongly basic anion exchanger was used to purify several radiolabelled nucleotides and nucleotide analogues that we needed with very high radiochemical purity for metabolic studies.

[U-^{14}C]ADP can be mentioned as an example of such a purification procedure. Storage at −20°C for several years resulted in partial decomposition of this labelled compound and it was necessary to remove the impurities before it could be used. The one-step purification procedure was performed as follows: 7.4 MBq of the sample (specific activity 13 GBq/mmol) were applied to a column of Separon HEMA-1000 Q in the formate form and purified using a 30-min linear gradient of ammonium formate from 0 to 0.3 mol/l with 0.06 mol/l formic acid. A 3-ml fraction corresponding to the elution of [U-^{14}C]ADP was collected, evaporated and co-dis-

TABLE I

CHROMATOGRAPHIC RESULTS FOR RIBONUCLEOTIDE SEPARATION USING THE STRONGLY BASIC ANION EXCHANGER SEPARON HEMA-1000 Q

Linear gradient of ammonium formate from 0 to 0.6 mol/l for 60 min. The mobile phase contained 0.06 mol/l formic acid. Flow-rate, 1.2 ml/min.

Ribonucleotide	t_R (min)	k'	N	HETP (μm)	R_s	A_s
CMP	4.9	1.0	3330	37		1.0
					9.8	
AMP	11.8	3.9	3090	39		1.1
					1.7	
UMP	13.7	4.7	2460	49		1.1
					2.3	
CDP	17.0	6.1	2510	48		1.3
					1.2	
GMP	18.8	6.8	1960	61		2.3
					4.0	
UDP	27.5	10.5	3140	38		1.6
					1.3	
ADP	29.7	11.4	3690	32		2.2
					1.1	
CTP	32.2	12.4	3990	30		2.1
					1.3	
GDP	35.0	13.6	3720	32		2.4
					2.3	
UTP	41.5	16.3	4240	28		2.3
					1.4	
ATP	46.0	18.2	4056	30		1.9
					1.3	
GTP	50.6	20.1	3530	34		3.0

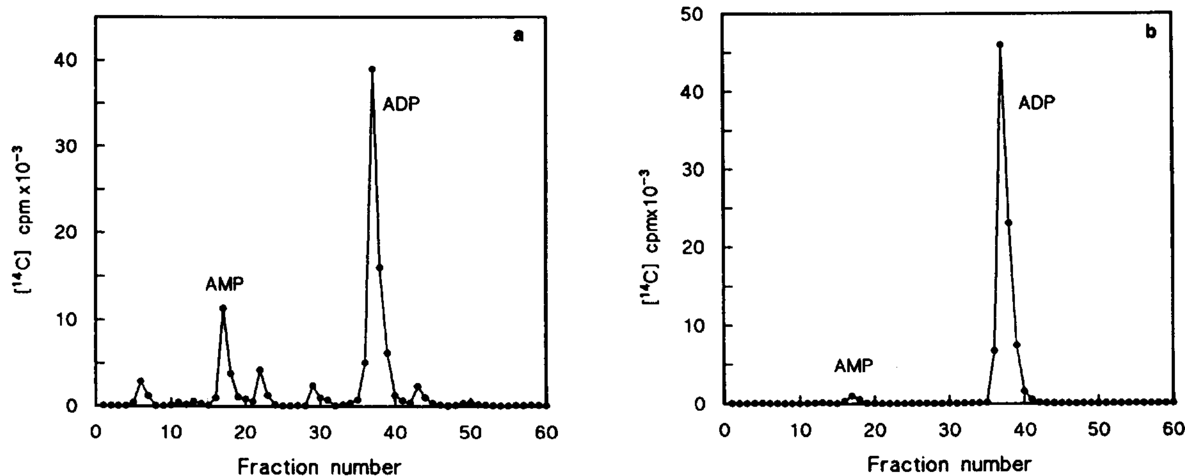

Fig. 6. Radiochemical purity of [U-^{14}C]ADP (a) before and (b) after purification on Separon HEMA-1000 Q. Analysis was carried out using ion-pair HPLC under the conditions described in Experimental.

tilled several times with water on a Speedvac to remove traces of the elution buffer components. The radiochemical purity of the sample was found to be higher than 98% using ion-pair HPLC. Fig. 6 shows the purity of [U-^{14}C]ADP before and after purification. Similar results were achieved in the purification of [U-^{14}C]AMP, [U-^{14}C]GMP and several ^{3}H- and ^{14}C-labelled nucleotide analogues.

Use of the volatile elution buffer system enables the purification to be carried out rapidly and simply and therefore this procedure is suitable especially for the purification of higher phosphates of nucleotides because of their lower stability.

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