Journal of Chromatography, 461 (1989) 259–266
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1509

ISOCRATIC REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF RIBONUCLEOTIDES, DEOXYNUCLEOTIDES, CYCLIC NUCLEOTIDES AND DEOXYCYCLIC NUCLEOTIDES

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SUMMARY

An isocratic reversed-phase high-performance liquid chromatographic system is described for the separation of a relatively large number (>20) of ribonucleotides, deoxynucleotides, cyclic nucleotides and deoxycyclic nucleotides. A 25 cm × 5 mm ODS-Hypersil (5 μm particle size) column was used with methanol–triethylammonium phosphate buffer as eluent. The effects of methanol content, pH, and ionic strength of the buffer on retention and resolution of the nucleotides have been studied. The applicability of the system was demonstrated by the analysis of nucleotides in cells and tissue extracts.

INTRODUCTION

Since Horváth *et al.*[1] reported the separation of nucleotides on a pellicular ion-exchange column, much of the early high-performance liquid chromatographic (HPLC) separation of nucleotides has been based on ion-exchange chromatography[2–5]. The technique is still widely usd and has been considerably improved[6–12] with the development of microparticulate, chemically bonded anion-exchangers. Reversed-phase[13–25] and reversed-phase ion-pair chromatography[26–33] were introduced later as alternatives to ion-exchage chromatography. The nucleotides are usually separated by gradient elution chromatography and, although isocratic systems have been described, they were only for the separation of a limited number of compounds. We believe further improvement in column efficiency, resolution, and speed of analysis is possible, particularly with reversed-phase chromatography, by manipulation of the mobile phase. The present paper describes the development and application of an isocratic system, capable of simultaneously separating a relatively large number of ribonucleotides, deoxynucleotides, cyclic nucleotides and deoxycyclic nucleotides.

EXPERIMENTAL

Materials and reagents

Nucleotides were from Sigma (Poole, U.K.). Triethylamine, orthophosphoric

0021-9673/89/$03.50

acid, perchloric acid, potassium carbonate, potassium hydroxide, dipotassium hydrogenphosphate and *p*-toluenesulphonyl chloride were of AnalaR grade from BDH (Poole, U.K.). Triethylamine was redistilled over *p*-toluenesulphonyl chloride before use. Methanol was of HPLC grade from Rathburn (Walkerburn, U.K.).

Extraction of nucleotides from cells and tissues

Nucleotides were extracted from cell suspension or freeze-clamped tissue homogenate into ice-cold 20% (w/w) perchloric acid and centrifuged at 2000 *g* for 10 min. The supernatant was adjusted to pH 6.0–6.5 with an ice-cold mixture of 4 *M* potassium hydroxide and 1 *M* potassium hydrogenphosphate or a saturated solution of potassium carbonate.

High-performance liquid chromatography

A Varian Assoc. (Walnut Creek, CA, U.S.A.) Model 5000 liquid chromatograph was used with a variable-wavelength detector (Varian UV-100), set at 254 nm. The 254–280 nm wavelength ratios were measured using two detectors in series. The separation was carried out on a 25 cm × 5 mm ODS-Hypersil (5 μm particle size) column (Shandon Southern, Runcorn, U.K.) with methanol in triethylammonium phosphate buffer as eluent at a flow-rate of 1 ml/min. The buffer was prepared by adjusting the pH of orthophosphoric acid with redistilled triethylamine. Buffers of various molarity and pH were made. Sample injection was via a Rheodyne (Cotati, CA, U.S.A.) 7125 injector, fitted with a 100-μl loop.

RESULTS AND DISCUSSION

The simultaneous analysis of a wide range of nucleotides in biological materials, *e.g.*, nucleotide profile, requires gradient elution chromatography. However, in many biomedical and biochemical applications, relatively few nucleotides are measured. A highly efficient isocratic system, capable of resolving the compounds of interest from interferences, should therefore be adequate for such applications. An isocratic system is attractive, because apart from being more reproducible, it also overcomes the major problem of base-line drift common to gradient elution chromatography of nucleotides.

Of all the HPLC systems described for the separation of nucleotides, reversed-phase chromatography offers greater potential for further improvement, particularly by exploiting the solute–solvent–stationary phase interactions. The separation of a standard mixture of ribonucleotides and cyclic nucleotides on ODS-Hypersil with methanol–83.3 m*M* triethylammonium phosphate (pH 6.0) (4:96, v/v) as eluent is shown in Fig. 1. The same system was used for the separation of deoxy and deoxycyclic nucleotides (Fig. 2). The capacity ratio (k') values are shown in Table I. The speed, resolution, and the relatively large number of compounds separated clearly demonstrate the superiority of the system over other isocratic systems for nucleotides. The improvement is mainly attributed to the use of triethylammonium phosphase as the eluent buffer. Acidic amine phosphate buffers have been shown to possess properties, such as masking of residual silanol groups and acceleration of proton equilibrium, which are particularly favourable to reversed-phase chromatography[34]. The choice of triethylammonium phosphate was dictated by these considerations, as it is expected to have similar chromatographic properties. Triethylamine is, in fact, a well-known silanol-masking agent.

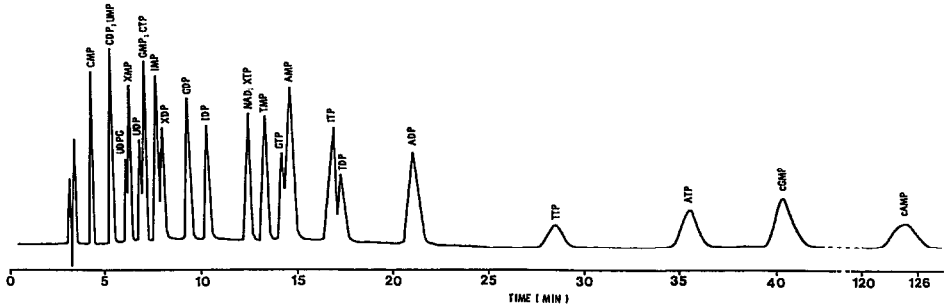


Fig. 1. Separation of a standard mixture of ribonucleotide and cyclic nucleotides. Column, ODS-Hypersil (25 cm × 5 mm I.D.); mobile phase, methanol–83.3 m*M* triethylamonium phosphate (4:96, v/v); flow-rate, 1 ml/min; detector, 254 nm.

The elution orders of cytidine < uridine < xanthosine < guanosine < inosine < thymidine < adenosine nucleotides and ribo < deoxyribo < deoxycyclic < cyclic nucleotides are in general consistent with the solvophobic theory proposed for reversed-phase chromatography with buffered eluents[35]. Thus, the more hydrophobic (less polar) nucleotides were retained longer than the less hydrophobic (more polar) ones. However, the elution order of nucleotide mono- < di- < tri-phosphates is the opposite of that expected and is similar to that observed for ion-exchange or ion-pair chromatography. This indicated a mixed retention mechanism. Ion pairing is more likely than ion-exchange chromatography, because triethylamine is an ion-pairing agent. Replacing triethylamonium phosphate with ammonium phosphate or ammonium acetate as the mobile phase buffer reverses the elution order to nucleotide tri- < di- < mono-phosphates.

Rapid separation of the more hydrophobic nucleotides can be achieved by increasing the organic modifier (methanol) content in the mobile phase (Fig. 3). The k' of cAMP, for example, was reduced from 38.2 to 8.2 when the methanol content was increased from 4 to 10% (v/v). The effect of methanol content on the k' of selected nucleotides is shown in Fig. 4. Increasing the methanol content does not necessarily imply a loss in resolution. Some nucleotide pairs, for example AMP and CTP, are actually better resolved at higher (6%) rather than lower (4%) methanol content. This is because, although increasing the methanol content decreases the k' of all nucleotides, the magnitude of this decrease is different for each nucleotide (Fig. 4), the triphosphate being more significantly affected than the di- and monophosphate nucleotides.

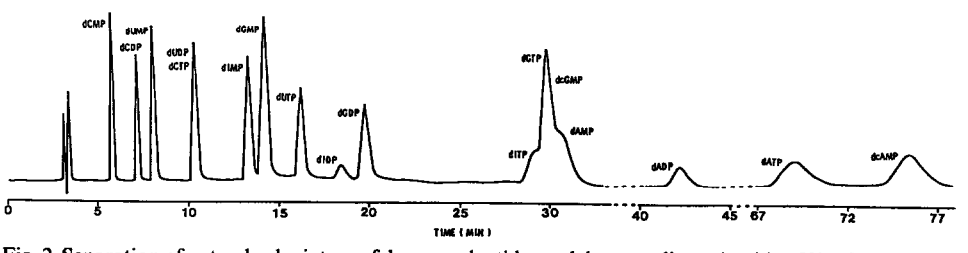


Fig. 2. Separation of a standard mixture of deoxynucleotides and deoxycyclic nucleotides. HPLC conditions as in Fig. 1.

TABLE I

CAPACITY RATIO (k') OF RIBONUCLEOTIDES, DEOXYNUCLEOTIDES, CYCLIC NUCLEOTIDES AND DEOXYCYCLIC NUCLEOTIDES ON 5-μm HYPERSIL-ODS WITH METHANOL–83.3 m*M* TRIETHYLAMMONIUM PHOSPHATE BUFFER (pH 6.0) (4:96, v/v) AS MOBILE PHASE

Compound	*Abbreviation*	*Capacity ratio (k')*
Cytidine 5'-monophosphate	CMP	0.4
Cytidine 5'-diphosphate	CDP	0.7
Uridine 5'-monophosphate	UMP	0.7
2'-Deoxycytidine 5'-monophosphate	dCMP	0.8
Uridine 5'-phosphoglucose	UDPG	0.9
Xanthosine 5'-monophosphate	XMP	1.0
Uridine 5'-diphosphate	UDP	1.2
2'-Deoxycytidine 5'-diphosphate	dCDP	1.2
Guanosine 5'-monophosphate	GMP	1.3
Cytidine 5'-triphosphate	CTP	1.3
Inosine 5'-monophosphate	IMP	1.4
2'-Deoxyuridine 5'-monophosphate	dUMP	1.5
Xanthosine 5'-diphosphate	XDP	1.5
Guanosine 5'-diphosphate	GDP	1.9
2'-Deoxyuridine 5'-diphosphate	dUDP	2.2
2'-Deoxycytidine 5'-triphosphate	dCTP	2.2
Inosine 5'-diphosphate	IDP	2.3
Nicotinamide adenine dinucleotide	NAD	2.9
Xanthosine 5'-triphosphate	XTP	2.9
Thymidine 5'-monophosphate	TMP	3.2
2'-Deoxyinosine 5'-monophosphate	dIMP	3.2
2'-Deoxyguanosine 5'-monophosphate	dGMP	3.4
Guanosine 5'-triphosphate	GTP	3.5
Adenosine 5'-monophosphate	AMP	3.6
2'-Deoxyuridine 5'-triphosphate	dUTP	4.1
Inosine 5'-triphosphate	ITP	4.3
Thymidine 5'-diphosphate	TDP	4.4
2'-Deoxyinosine 5'-diphosphate	dIDP	4.8
2'-Deoxyguanosine 5'-diphosphate	dGDP	5.2
Adenosine 5'-diphosphate	ADP	5.6
Thymidine 5'-triphosphate	TTP	8.0
2'-Deoxyinosine 5'-triphosphate	dITP	8.1
2'-Deoxyguanosine 5'-triphosphate	dGTP	8.3
2'-Deoxyguanosine 3':5'-cyclic monophosphate	dcGMP	8.3
2'-Deoxyadenosine 5'-monophosphate	dAMP	8.5
Adenosine triphosphate	ATP	10.1
Guanosine 3':5'-cyclic monophosphate	cGMP	11.6
2'-Deoxyadenosine 5'-diphosphate	dADP	12.3
2'-Deoxyadenosine 5'-triphosphate	dATP	20.7
2'-Deoxyadenosine 3':5'-cyclic monophosphate	dcAMP	22.6
Adenosine 3':5'-cyclic monophosphate	cAMP	38.2

The retention and resolution of the nucleotides may also be controlled by adjusting the pH of the buffer. Maximum retention and resolution of the purine nucleotides were between pH 4.5–6.0 (Fig. 5). The ionic strength of the buffer also affected retention and resolution (Fig. 6) but to a lesser extent than the pH. Buffers of between 80–85 m*M* are recommended for the present system.

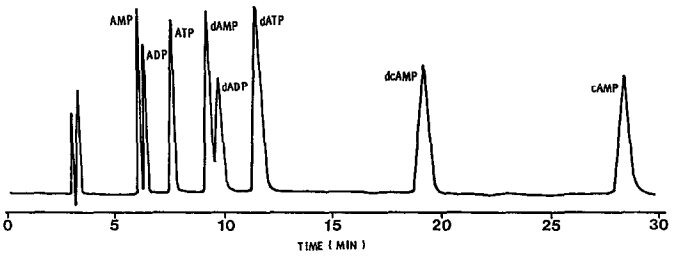


Fig. 3. Rapid separation of adenine nucleotides. Mobile phase, methanol–83.3 m*M* triethylammonium phosphate (pH 6.0) (10:90, v/v). Other HPLC conditions as in Fig. 1.

The applicability of the method is demonstrated by the analysis of nucleotides in cells and tissue extracts (Fig.7). The major nucleotides were clearly separated from impurities. The peaks were identified by chromatography after addition of standards and confirmed by absorbance ratio measurements at 254 and 280 nm.

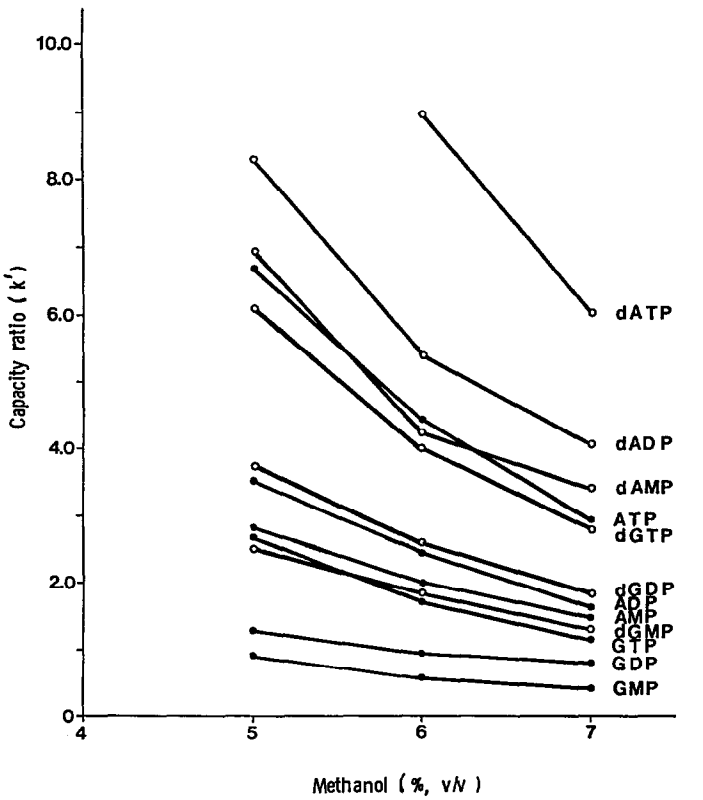
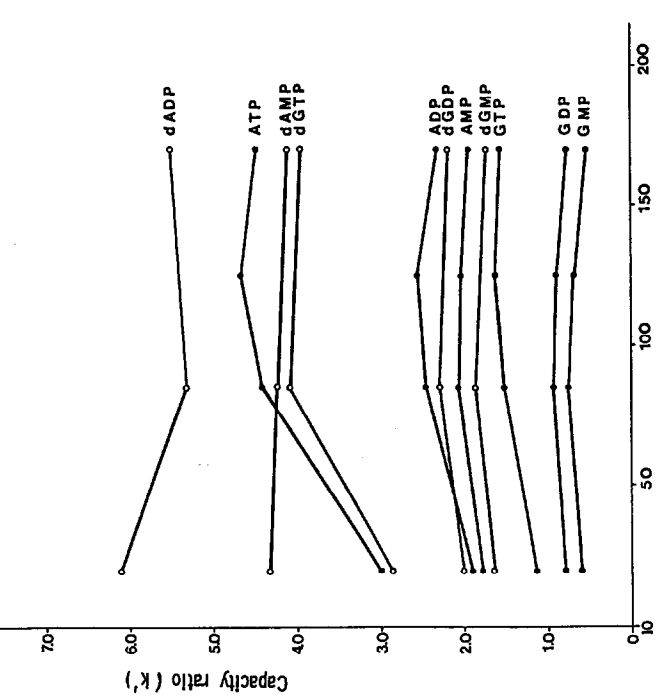


Fig. 4. Effect of methanol content on the retention and resolution of nucleotides. Mobile phase, methanol-83.3 m*M* triethylammonium phosphate (pH 5.0).



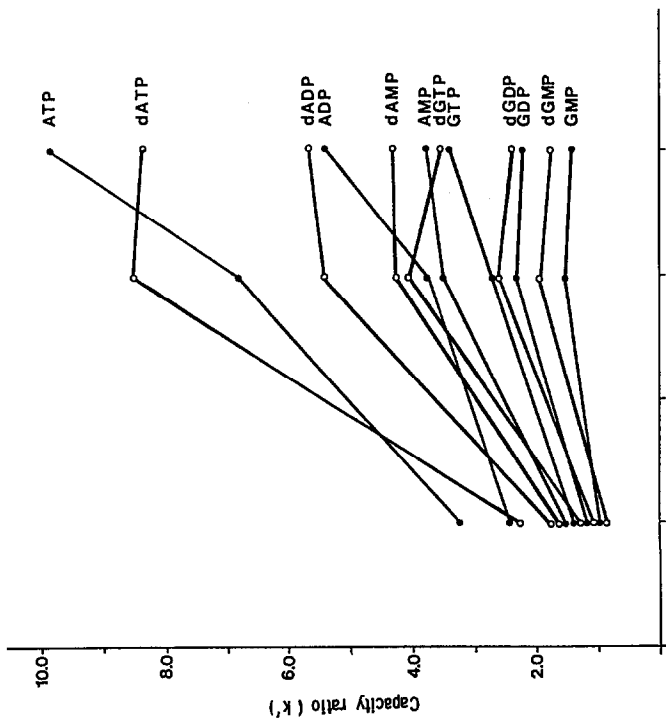


Fig. 5. Effect of pH on capacity ratios of nucleotides. Mobile phase, methanol–83.3 m*M* triethylammonium phosphate (6:94, v/v).

Fig. 6. Effect of triethylammonium phoshate buffer concentration on the retention of nucleotides. Mobile phase, methanol triethylammonium phosphate (pH 5.0) (6:94, v/v).

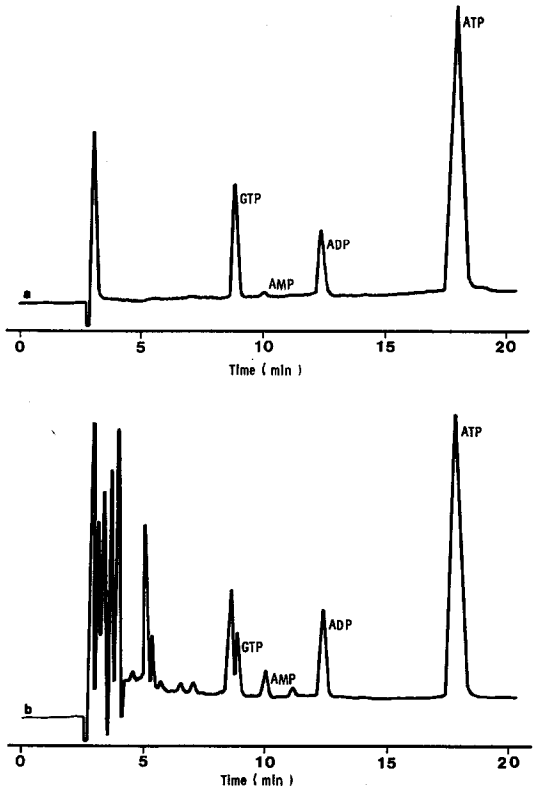


Fig. 7. Separation of nucleotides in (a) rat brown adipose tissue and (b) human lymphocytes. Column, ODS-Hypersil; eluent, methanol–83.3 m*M* triethylammonium phospate (pH 6.0) (6:94, v/v).

CONCLUSIONS

Triethylammonium phosphate is an excellent mobile phase buffer for reversed-phase chromatography of nucleotides. Isocratic systems with methanol in triethylammonium phosphate of controlled pH and ionic strength as mobile phases allowed rapid separation of more than twenty nucleotides without base-line drift. The retention mechanism is a mixed one, involving hydrophobic interaction and ion-pair formation. The system is suitable for a wide range of biomedical and biochemical applications.

ACKNOWLEDGEMENT

We are grateful to Mrs. Sheila Kingsley for secretarial assistance.

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