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Separation of ribonucleotides, deoxynucleotides, cyclic nucleotides and deoxycyclic nucleotides by reversed-phase high-performance liquid chromatography

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Abstract: A reversed-phase gradient elution system with methanol-triethylammonium phosphate buffer (83.3 mM, pH 6.0) as eluent on C_{18}-bonded silica is described for the separation of 38 ribonucleotides, deoxynucleotides, cyclic nucleotides and deoxycyclic nucleotides in less than 33 min. The retention of the nucleotides can be precisely controlled by adjusting the pH, buffer concentration and methanol content in the mobile phase. The system is especially useful for the analysis of low levels of cyclic nucleotides in cells and tissues.

Keywords: *Reversed-phase high-performance liquid chromatography; ribonucleotides; deoxynucleotides; cyclic nucleotides; deoxycyclic nucleotides.*

Introduction

The analysis of nucleotides in cells and tissues is important in biochemistry and in clinical medicine. Nucleotides are implicated in diseases such as arthritis, renal failure, cardiovascular disorders [1], malignancy [2, 3] and immunodeficiency diseases [4, 5].

The nucleotides are usually separated by ion-exchange [6–8] or reversed-phase chromatography [9, 10]. Ion-pairing [11, 12] and zwitterion-pairing [13] systems have also been described. Of these, reversed-phase chromatography is the most versatile technique and has more scope for further improvements in the speed of analysis, column efficiency and resolution.

The mobile phases most commonly used in high-performance liquid chromatography (HPLC) of nucleotides are KH_2PO_4, NaH_2PO_4 or $NH_4H_2PO_4$ buffers with methanol as the organic modifier. These systems, although useful for the analysis of a limited number of nucleotides, are inadequate for the simultaneous resolution of ribonucleotides, deoxynucleotides, cyclic nucleotides and deoxycyclic nucleotides which may be required in biochemical and biomedical applications.

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The present paper describes a simple gradient elution system, with triethylammonium phosphate and methanol, for the rapid and effective separation of nucleotides. The method is applicable to the analysis of nucleotides in cells and tissues.

Experimental

Materials and reagents

Nucleotides were from Sigma Chemical Co., Poole, Dorset, UK. Triethylamine, orthophosphoric acid, perchloric acid, potassium hydroxide and dipotassium hydrogen phosphate were AnalaR grade from BDH Chemicals, Poole, Dorset, UK. Triethylamine was redistilled over *p*-toluenesulphonyl chloride before use. Methanol was HPLC grade from Rathburn Chemicals, Walkerburn, Peebleshire, UK.

Nucleotide extraction from cells and tissues

Nucleotides were extracted from cell suspension or tissue homogenate into ice-cold 20% (w/v) perchloric acid and centrifuged at 2000 ***g*** for 10 min. The clear supernatant was adjusted to pH 6.0–6.5 with an ice-cold mixture of 4M KOH and 1M K_2HPO_4 or a saturated solution of K_2CO_3.

HPLC

A Varian Associates (Walton-on-Thames, Surrey, UK) model 5000 liquid chromatograph was used. Injection was via a Rheodyne 7125 injector (Berkeley, CA, USA) fitted with a 100 μl loop. A variable-wavelength UV detector (Varian UV-100) set at 254 nm was used. The separation was carried out on 25 cm × 5 mm columns packed with ODS-Hypersil [5 μm spherical silica chemically bonded with octadecylsilyl groups (Shandon Southern, Runcorn, Cheshire, UK)]. The column packing had a surface area of 170 m^2 g^{-1} with 10% carbon leading and was end-capped with trimethylsilyl groups.

The solvents for the gradient elution were 1% (v/v) methanol in 83.3 mM triethylammonium-phosphate buffer pH 6.0 (solvent A) and 20% (v/v) methanol in 83.3 mM triethylammonium phosphate buffer pH 6.0 (solvent B). The buffer was prepared by adjusting the pH of 83.3 mM orthophosphoric acid to 6.0 with redistilled triethylamine (about 12.5 ml). Buffers of various molarity and pH were similarly made up.

The elution program was as follows: 0–20.0 min, 0% B (100% A)–45% B (55% A); 20.1–28.0 min, 45% B (55% A)–100% B (0% A); 28.1–33.0 min, 100% B (0% A). The flow rate was 1 ml min^{-1}.

A simplified system in which solvent A was triethylammonium phosphate buffer and solvent B 15% (v/v) methanol in the buffer was used for retention behaviour study. A 20-min linear gradient from 100% A (0% B) to 0% A (100% B) was used.

Peak identification

Peaks in biological extracts were identified by cochromatography with pure standards and by the characteristic absorbance ratio at 254 and 280 nm [9].

Results and Discussion

Separation of nucleotides by reversed-phase HPLC

It has been shown that in reversed-phase HPLC of nucleotides retention is affected by solute–stationary phase–mobile phase interactions which involved the organic modifier

(methanol), the hydrocarbonaceous chain and the accessible residual silanol groups of the stationary phase [10]. The nature of the mobile phase is therefore an important parameter for achieving good chromatography of the nucleotides. Acidic amine phosphate buffers have been studied by Melander *et al.* [14], and their superior chromatographic properties were clearly demonstrated. Although triethylammonium phosphate itself was not included in their study, its important properties such as masking of residual silanols and ability to accelerate the rate of proton equilibrium in the chromatographic process are expected to be similar.

The separation of a standard mixture of ribonucleotides, deoxynucleotides, cyclic nucleotides and deoxycyclic nucleotides by the methanol–triethylammonium phosphate system is shown in Fig. 1. The separation of 38 compounds required just 33 min. This is much faster than other reversed-phase systems reported [9] for the separation of a small number (about 20) of nucleotides. The system is also superior to ion-exchange chromatography in terms of speed and resolution and does not suffer as much from detector base-line drift common to ion-exchange HPLC of nucleotides [7, 8]. The ability of triethylammonium phosphate to mix with methanol in all proportions is another advantage as it eliminates the possibility of salt crystallization during gradient elution, often a problem with the relatively insoluble inorganic phosphates.

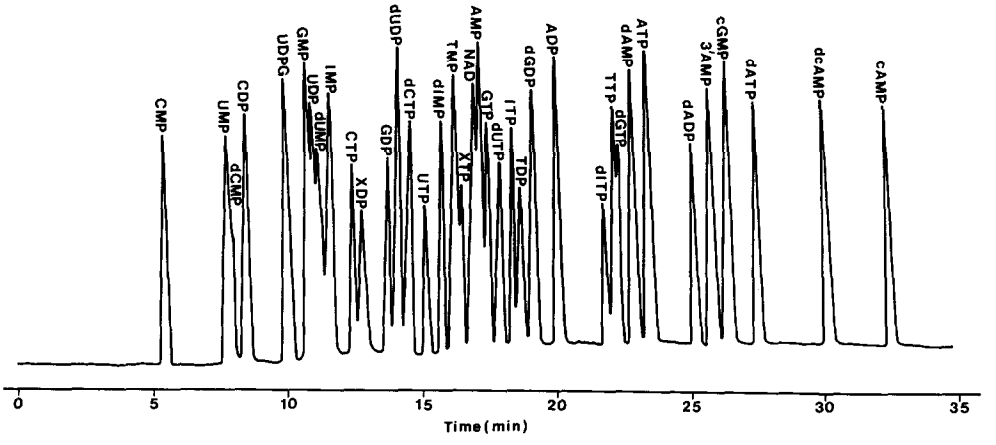

Figure 1
Separation of a standard mixture of nucleotides. Column, ODS-Hypersil (25 cm × 5 mm). Gradient mixture: 1% (v/v) methanol in 83.3 mM triethylammonium phosphate pH 6.0 (solvent A) and 20% (v/v) methanol in the same buffer (solvent B). Elution program: 0–20.0 min, 0% B (100% A)–45% B (55% A); 20.1–28.0 min, 45% B–100% B; 28.1–33.0 min, 100% B. Flow rate, 1 ml min^{-1}. Detector, UV 254 nm.

Control of retention by pH adjustment

The dominant retention mechanism in reversed-phase chromatography is hydrophobic interaction between the solutes and the hydrocarbonaceous stationary phase surface. Nucleotides are ionogenic compounds and their state of ionization and therefore the relative hydrophobicity is pH-dependent. The effect of pH on the retention of the nucleotides is shown in Fig. 2, a plot of the pH of the eluent against the capacity ratios (κ') of the compounds. κ' decreased with decreasing pH. This behaviour is due to the increasing ionization of the nitrogen of the nucleotides as the pH is lowered with the consequent decrease in hydrophobicity of the compounds.

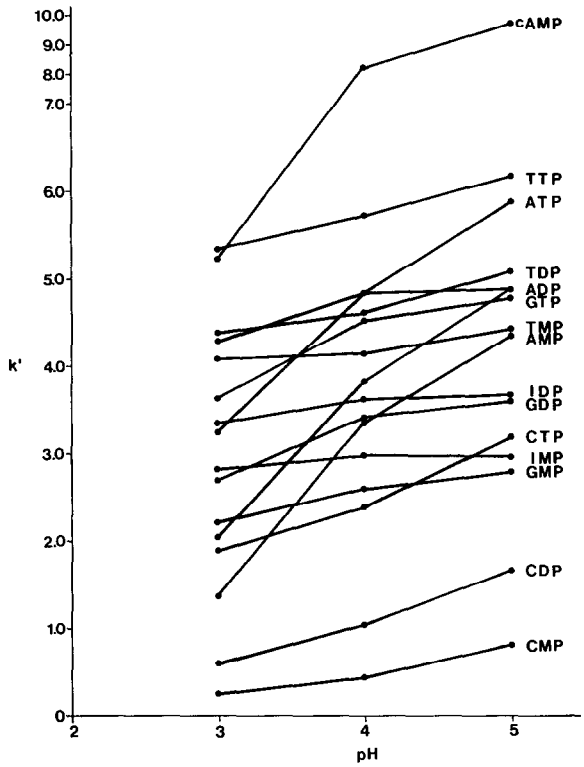

Figure 2
Effect of pH on capacity ratios of nucleotides. Column, ODS-Hypersil (25 cm × 5 mm). Gradient mixture: 83.3 mM triethylammonium phosphate pH 3, 4 and 5 (solvent A) and 15% (v/v) methanol in the same buffer (solvent B). Elution, 20-min linear gradient from 0% B to 70% B followed by isocratic elution at 70% B. Flow rate, 1 ml min^{-1}. Detector, UV 254 nm.

Retention control by altering buffer concentration

The effect of buffer concentration (ionic strength) on the retention (Fig. 3) is that expected for hydrophobic chromatography where retention increases with increasing buffer concentration [15]. An exception was observed for the cytidine nucleotides where κ' increased to a maximum at 83.3 mM and decreased at higher buffer concentrations. This may be a result of an ion-exchange or an ion-pairing mechanism operating in addition to the hydrophobic interaction. It is well known that increasing the ionic strength of a buffer decreased the κ' in ion-exchange and ion-pair chromatography [13].

Control of retention by adjusting the methanol content

The methanol content in the mobile phase significantly affected the retention of nucleotides; typically, increasing methanol content decreased the κ'. However, mixed retention mechanisms can occur under certain conditions and increasing the methanol content actually caused an increase in κ' of some nucleotides, especially the triphosphate nucleotides [10]. In the present study the methanol content was kept at a maximum of 20% (v/v) and deviation from the usual reversed-phase behaviour was not observed.

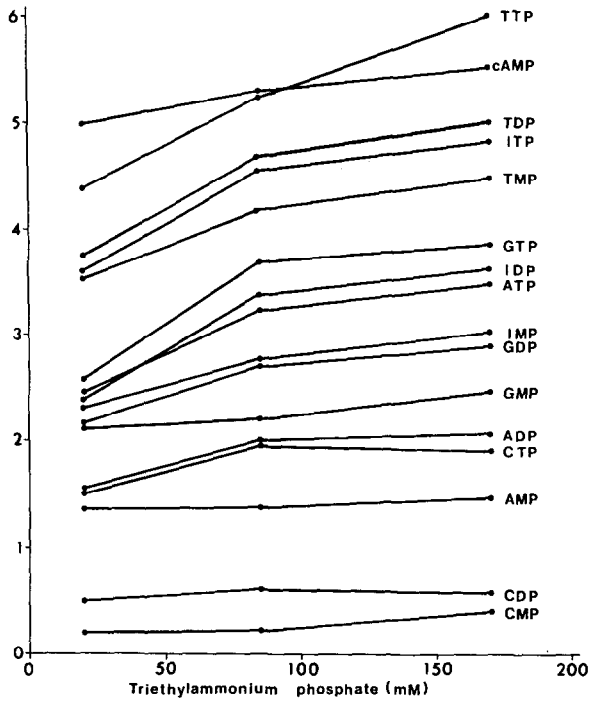

Figure 3
Effect of triethylammonium phosphate buffer concentration on the retention of nucleotides. Gradient mixture: 16.6, 83.3 and 166.6 mM triethylammonium phosphate pH 3.0 (solvent A) and 15% (v/v) methanol in the same buffer (solvent B). Other HPLC conditions as in Fig. 2.

Example of applications

The applicability of the present system is demonstrated by the separation of nucleotides in human erythrocytes (Fig. 4). All the important nucleotides were well resolved. The presence of a much higher concentration of ATP in the red cells required a reduction of the detector sensitivity from 0.16 to 1.28 absorbance units full scale (a.u.f.s.) in order to keep the peak on scale. The synthetic 3′-AMP is an ideal internal standard for the quantitative determination of nucleotides in cells and tissue extracts.

Conclusion

Gradient elution with triethylammonium phosphate–methanol mobile phases on C_{18}-bonded reversed-phase columns separated 38 ribonucleotides, deoxynucleotides, cyclic nucleotides and deoxycyclic nucleotides in under 33 min. The versatility and flexibility of the system allows for optimization of separation and detection according to the nature of application required. The system is especially useful for the analysis of cyclic nucleotides. These compounds, being more hydrophobic, were eluted at the end of the chromatogram, thus allowing the sensitivity of the detector to be greatly increased for their detection because of the low concentration present in cells and tissues. This is not possible with other HPLC systems where cyclic nucleotides are eluted at the front end or at the middle of the chromatogram and attempts to increase the detector sensitivity will lead to severe interference by compounds present in much higher concentrations.

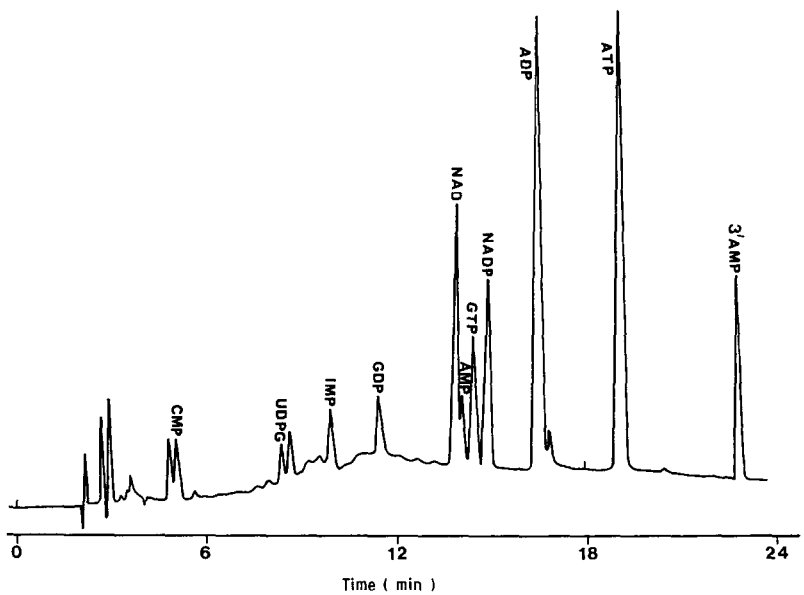

Figure 4
Separation of nucleotides in human erythrocytes. Column ODS Hypersil (25 cm × 5 mm). Gradient mixture : 1% (v/v) methanol in 83.3 mM triethylammonium phosphate pH 6.0 (Solvent A) and 20% (v/v) methanol in the same buffer (Solvent B). Elution program: 0–20.0 min, 0% B (100% A)–55% B (45% A); 20.1–28.0 min, 55% B–100% B. Flow rate, 1 ml min^{-1}. Detector, UV 254 nm set at 0.16 a.u.f.s. and changed to 1.28 a.u.f.s. at 18 min.

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