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DETERMINATION OF ADENOSINE RIBO- AND DEOXYRIBONUCLEOTIDES AS THEIR 1-N^6-ETHENO DERIVATIVES BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The chromatographic resolution of fluorescent 1-N^6-etheno derivatives of adenine and adenosine ribo- and deoxyribonucleotides on reversed-phase columns has been optimised by a systematic study of the effect of ion-pair concentration, pH, eluent molarity and methanol concentration. Using tetrabutylammonium hydrogen sulphate as the ion-pair separation of up to eight derivatives could be achieved in *ca.* 10 min. Conditions of derivatisation with chloroacetaldehyde have been investigated in order to reduce hydrolysis of the nucleotides.

INTRODUCTION

Although UV detection at 240–270 nm is universal for nucleic acid compounds in many applications, it is limited by poor sensitivity and poor selectivity. Gradient elution is often necessary[1,2] and this in turn further reduces sensitivity. The need for systems able to measure selected groups of compounds at high sensitivity and with shorter analysis times exists, for example, in the determination of deoxyribonucleotides within cells and the quantitation of cyclic nucleotides. The determination of deoxyribonucleotides in biological extracts is particularly difficult, since they are normally present at *ca.* 1% of the level of the corresponding ribonucleotides. To overcome this difficulty, methods involving the removal of ribonucleotides prior to high-performance liquid chromatography (HPLC) have been devised. Selective degradation of the 2'3'-*cis*-diol of ribonucleotides with periodate and methylamine has been employed and various workers have combined this with UV detection to measure deoxynucleotides but sensitivity is poor[3–5].

Haloacetaldehydes react with adenine- and cytidine-containing compounds to form fluorescent 1-N^6-etheno (ε) derivatives[6,7]. The reaction has been employed for the determination of total adenine compounds in cells[8]. Methods involving pre-column derivatisation and separation of these derivatives of adenine, adenosine[9–11] and cAMP[12] by HPLC have been published. The method is extremely sensitive being able to determine less than 1 pmol of the derivatives. The separation of the ε derivatives of ATP, ADP, AMP and cAMP by anion exchange has been reported, but the

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analysis time was 40 min (ref. 13). Recently, the ε derivatives of the adenosine nucleotides have been separated by reversed-phase HPLC both isocratic[14] and with gradient elution[15]. The strongly ionic nature of ε-nucleotides means that their chromatography is not totally satisfactory by reversed-phase.

The separation of adenine, adenosine-containing ribo- and deoxyribonucleotides and cAMP by reversed-phase HPLC in the presence of tetrabutylammonium ion has been achieved[16]. This paper examines some of the chromatographic variables, such as pH, ion-pair and methanol concentrations, the optimisation of which are essential for a reliable assay. The reaction conditions have also been investigated in order to minimise the hydrolysis of the nucleotides during derivatisation.

MATERIALS AND METHODS

Chemicals

Nucleotides, nucleosides and bases, sodium periodate, rhamnose, methylamine, sodium ethylenediaminetetraacetate (EDTA) and tetrabutylammonium hydrogen sulphate (TBAHS) were obtained from Sigma (Poole, U.K.). HPLC-grade methanol was from Rathburn (Walkerburn, U.K.). Chloroacetaldehyde was obtained either from Fluka (via Fluorochem, Stalybridge, U.K.) as a 55% solution which was further diluted to 10% or by distillation from 5% sulphuric acid of the dimethyl acetal (Sigma), and the distillate (b.p. 88–90°C) was diluted with water to a final concentration of 10%. At 4°C this solution keeps for up to six months.

Chromatography

The HPLC system consisted of an ACS 500 pump (ACS, Macclesfield, U.K.), a Rheodyne valve injector with a 20-μl loop (Anachem, Luton, U.K.), and a Kratos FS970 fluorescence detector, equipped with a deuterium lamp and a >387-nm emission filter (Kratos, Manchester, U.K.). The detector output was recorder on either a 0–10 mV potentiometric recorder or a HP 3390A integrator (Hewlett Packard, Beaconsfield, U.K.). Chromatographic columns 100 × 4.6 mm I.D. (HETP, Macclesfield, U.K.) were packed in the laboratory according to the manufacturer's instructions with 3-μm ODS-Hypersil (Shandon, Runcorn, U.K.). In the final procedure, the column was eluted at 1.2 ml/min using a 50 m*M* ammonium acetate buffer (pH 5.5) containing 0.2 m*M* TBAHS and 1 m*M* sodium EDTA mixed with methanol (9:1). The purity of the nucleotides was checked using a previously described anion-exchange gradient system[17].

Formation of 1-N^6-etheno derivatives

In the final procedure 0.1 ml of nucleotide-containing sample was mixed with 1 ml of 0.2 *M* ammonium acetate buffer (pH 6.0) containing 4 m*M* sodium EDTA in either stoppered glass tubes or 1.5-ml capped polypropylene vials. 50 μl chloroacetaldehyde solution was added, and the mixture was stirred. The tubes were placed in a 95°C water bath for 10 min. At the end of that period, they were rapidly cooled, and 20 μl was injected into the HPLC column.

Periodate oxidation

The oxidation of ribonucleotides was described by Garrett and Santi[3] and

optimised by Tanaka *et al.*[5]. A 20-μl volume of 0.2 *M* sodium periodate was added to 80 μl of a solution containing either underivatised nucleotides or ε-nucleotides. The mixture was stirred and centrifuged for 20 s followed by incubation at 37°C for 2 min. Then, 2 μl of 1 *M* rhamnose was added followed, by 30 μl 4 *M* methylamine (neutralised to pH 6.5 with orthophosphoric acid). The mixture was stirred, incubated at 37°C for 30 min and then cooled on ice. The final reaction mixture was injected directly into the column.

RESULTS

The excitation spectrum, determined by repeated injections of 100 pmol of ε-adenosine, is shown in Fig. 1. With the deuterium lamp of the detector the excitation maximum was 212 nm. This uncorrected value is considerably lower than that previously reported using different detectors. Other research workers have used wavelengths of *ca.* 280 nm, which give only 7% of the response obtained at 212 nm. Unfortunately, for high-sensitivity analyses this increase is not maintained. Only a 20% increase in sensitivity was found at 232 nm when 5 pmol of ε-adenosine was repeatedly injected at the maximum sensitivity that could be offset by the fluorometer at each wavelength (Fig. 1). Of the available emission filters, the 389-nm pass filter gave the best response.

With the particular reversed-phase packing employed, the pH, ion-pair concentration, methanol concentration and buffer ionic strength were systematically varied while holding the other three variables constant at approximately their optimum value. The changes in retention with variations in the concentration of TBAHS and pH are shown in Fig. 2a and b, respectively. Optimum retention of the ε-nucleotides relative to the nucleotides and bases, was at 0.2–0.3 m*M* TBAHS when the pH of the eluent was 5.5. Increasing the methanol concentration as expected reduced the retention times in an approximately linear manner but did not significantly change the selectivity. Varying the molarity of the buffer also had little effect. The inclusion of EDTA in the eluent led to an improvement in column performance and reduced retention times by approximately 5%. This beneficial improvement was probably due to reduced metal chelation by the nucleotide side chain.

Using the optimum buffer composition, the retention characteristics of a number of 6-aminopurines were determined and are given in Table I. Fig. 3 shows a typical standard chromatogram, obtained by injecting a mixture of ε compounds equivalent to approximately 25 pmol of each derivative. The impurity peak at time 6.8 min was present in all samples. The size of both this peak and the frontal peaks increased with the age of the chloroacetaldehyde solution and when a significant excess of chloroacetaldehyde was employed. The use of fresh reagent at a 10% dilution minimised the size of this peak.

The optimum pH for the reaction was determined by incubating an equimolar mixture of adenosine, ATP, ADP and AMP in buffers of varying pH for a reaction time of 30 min. Maximum yield as determined by the sum of the integrator peak occurred at pH 5.5. With increasing pH the solutions became intensely yellow and, on standing, a fine black precipitate was observed to form.

Nucleotides, particularly nucleoside triphosphates, are readily hydrolysed at acid pH and elevated temperatures. In order to minimise hydrolysis of nucleotides

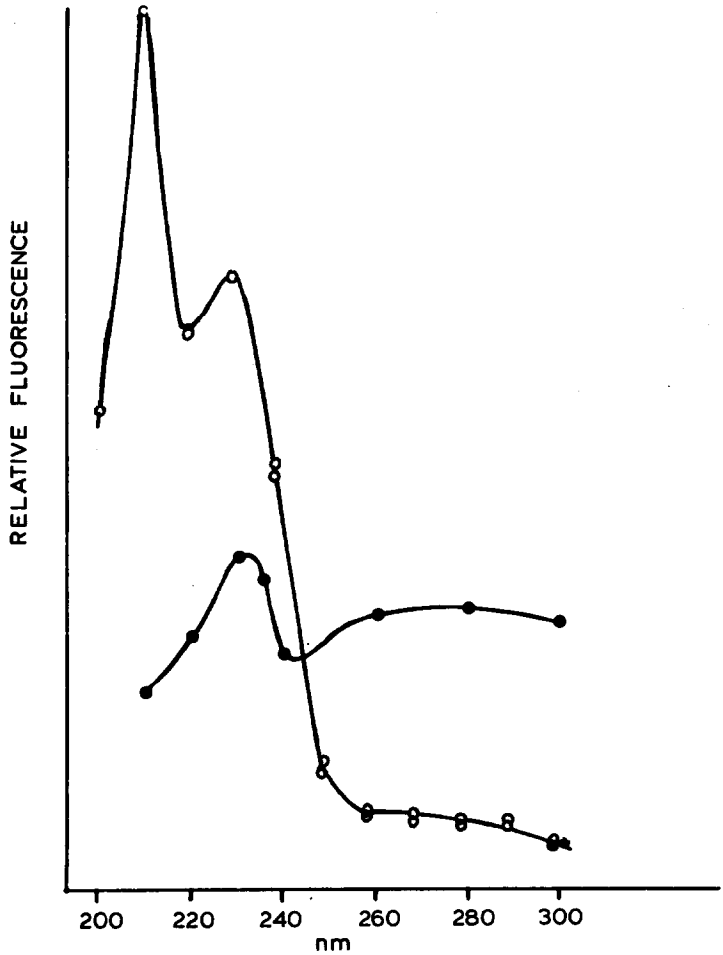

Fig. 1. Excitation spectrum (O–O) of 1-N^6-etheno adenosine in Kratos FS 970 fluorometer with deuterium lamp. Maximum sensitivity curve (●–●) obtained by assaying peak of a 5-pmol injection at the maximum sensitivity of detector.

during derivatisation the percent destruction of ATP was studied for various pH values and time courses at 95°C. Over the pH range 3.5 to 7.5 ATP was at least 40% hydrolysed after 45 min. A reasonable compromise between yield and hydrolysis was achieved by using a buffer (pH 6.0) at 95°C for just 10 min. The ionic composition of the buffer also affected both the fluorescence yield and the present hydrolysis (Table II). Incorporation of EDTA into the reaction buffer increased the yield, gave a more uniform yield with the three buffers tested, and approximately halved the hydrolysis, particularly for the acetate buffer. Since acetate buffer also gave the smallest frontal peak, it was chosen for the final derivatisation buffer. Once the derivatives are formed, they are stable at room temperature for at least a week without further hydrolysis of the nucleotide compounds.

When the above conditions for both the derivatisation and the chromato-

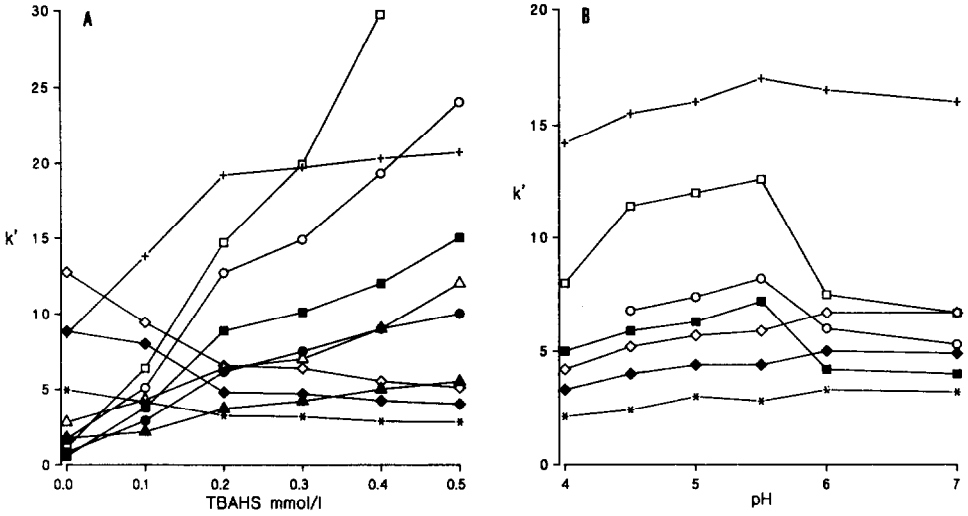

Fig. 2. Variation in k' for a mixture of ε derivatives on a 100 × 4.6 mm I.D. column of 3 μm ODS-Hypersil. Conditions as in Materials and methods, except (A) tetrabutyl ammonium ion concentration variable, (B) pH variable. Key to symbols: ■, ε-ATP; ●, ε-ADP; ▲, ε-AMP; ◆, ε-adenosine; □, ε-deoxyATP; ○, ε-deoxyADP; △, ε-deoxyAMP; ◇, ε-deoxyadenosine; *, ε-adenine; +, ε-cAMP.

graphy were used, the results for the tested compounds were linear over a wide range (1–500 pmol injected). Maximum sensitivity (S/N = 2) varied with the particular compound and the exact chromatographic conditions but ranged from 200 fmol injected for adenosine to 1 pmol injected for dATP.

The system developed has been employed to quantify nucleotides and related compounds in acid extracts of biological specimens. In practice it has been found preferable to degrade the ε derivatives rather than the original extract while leaving the deoxyribonucleotide derivatives intact since one derivatisation allowed the determination of both the deoxyribo- and ribo nucleotides.

TABLE I

RETENTION DATA FOR 1-N^6-ETHENO ADENINE COMPOUNDS

For chromatographic conditions see Materials and methods, k' = capacity factor.

Original compound	k'	*Original compound*	k'
AMP	3.14	NADP	3.40
ADP	4.09	DeoxyAMP	5.08
ATP	7.09	DeoxyADP	8.46
Adenine	2.99	DeoxyATP	13.4
Adenosine	4.84	Deoxyadenosine	6.43
cAMP	18.56		

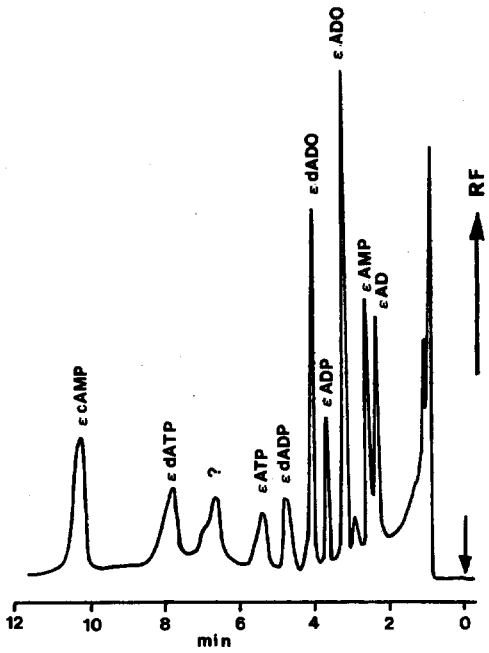

Fig. 3. Standard chromatogram, showing ion-pair reversed-phase liquid chromatography of a mixture of ε derivatives of adenine compounds. Initially, 25 pmol of each sample injected except deoxyATP (50 pmol) and adenosine and deoxyadenosine (12.5 pmol).

DISCUSSION

The chromatographic characteristics of some 1-N^6-etheno derivatives of 6-aminopurine in the presence of TBAHS is similar to that studied in detail for the parent compound by Perrone and Brown[18]. The major difference is that a signifi-

TABLE II

EFFECT OF BUFFER ON HYDROLYSIS OF ATP BY INCUBATION AT 95°C FOR 10 min

Buffer (pH 6.0)	*Yield (%)**	*Final composition (%)***		
		ATP	*ADP*	*AMP*
Starting composition		97	3	0
0.2 *M* ammonium acetate	54	90	8.9	1
0.2 *M* ammonium acetate plus 4 m*M* EDTA	100	95	4.9	0
0.2 *M* potassium phosphate	79	90	9.5	0.5
0.2 *M* potassium phosphate plus 4 m*M* EDTA	85	93	6.3	0
0.2 *M* sodium citrate	91	90	9.5	0
0.2 *M* sodium citrate plus 4 m*M* EDTA	86	94	5.2	0

* Expressed as percent of ammonium acetate plus EDTA.
** Expressed as percent of total integrated peak area.

cantly lower concentration of ion-pair was necessary for the derivatives (0.2 against 2 m*M*). This finding appears to be related to the particular reversed-phase packing material used in our study rather than to the derivatives, because when the same material was used for the parent nucleotides they were also resolved at these low concentrations[19]. The flexibility of the ion-pair system in achieving the resolution of both the adenosine nucleotides and other adenine compounds in a single run is a major advantage over the simple reversed-phase chromatography used by Preston[14] or Levitt *et al.*[15]. The nucleotides are well retained on the column rather than being eluted early, although this retention leads to some loss of sensitivity. Using the chromatographic data presented here, it is possible to optimise conditions for the measurement of any single compound to give maximum sensitivity in the shortest possible analysis time. During the course of this study, Ramos-Salazar and Baines[20] reported a similar procedure for adenosine ribonucleotides alone.

Other workers have used chloroacetaldehyde to derivatise nucleotides, but several derivatisation procedures have been published without regard to the possibility of hydrolysis. Preston[14] used 30 min in a boiling water bath but did not check for hydrolysis. Levitt *et al.*[15] used 40 min at 80°C and checked for the overall recovery of their system including tissue extraction, by addition of radiolabelled ATP. They found a recovery of 59.5% but did not comment on any hydrolysis. Ramos-Salazar and Baines[20] returned to the derivatisation of Barrio *et al.*[6], *i.e.* 24 h at 37°C, in order to minimise hydrolysis and reported that 19, 5 and 9% less ε-AMP, ε-ADP and ε-ATP respectively was formed than at 80°C for 40 min. In this present study the rate of hydrolysis has been more thoroughly examined and the percent destruction has been reduced significantly by lowering the incubation time, using a higher pH and including EDTA in the buffers. It must also be appreciated that using long incubation times at elevated temperatures in order to measure other adenine derivatives, *e.g.*, adenosine in tissues, may give increased values due to nucleotide degradation.

Recently, it has been reported[13] that bromoacetaldehyde can replace chloroacetaldehyde in the reaction and gives a 20% improvement in the yield of the nucleotide derivatives, since its optimum reaction conditions are 15 min at 80°C. Such conditions should reduce the hydrolysis of the nucleotides still further. Unfortunately, bromoacetaldehyde is not commercially available. Attempts to use a laboratory-synthesised reagent have not, so far, achieved a significant improvement in performance.

The determination of deoxyribonucleotides by UV detection and periodate oxidation is limited by sensitivity. The proposed method will only determine deoxyadenine compounds, but the sensitivity is better than that for UV detection alone.

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