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EVALUATION OF MICROPARTICLE CHEMICALLY BONDED REVERSED-PHASE PACKINGS IN THE HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF NUCLEOSIDES AND THEIR BASES*

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SUMMARY

The reversed-phase partition mode of high-pressure liquid chromatography was used for the analysis of seven of the naturally occurring nucleosides and their bases. With microparticle chemically bonded packings, nucleosides and their bases can be quantitatively determined in the presence of nucleotides in 30 min with high sensitivity, accuracy, and reproducibility. Peaks in chromatograms of cell extracts were identified by absorbance ratios and enzymatic peak shift methods. Applications of this technique to biochemical studies are reported.

INTRODUCTION

Many procedures have been used for the analysis of nucleosides and bases by high-pressure liquid chromatography (HPLC)[1–17]. The majority of these techniques were used for the separation of hydrolysates of DNA and RNA; thus the only compounds of interest in the samples were either the nucleoside or base pairs. Other analyses were developed to separate all the UV absorbing constituents in urine[18–20]. However, the time required for these analyses prevents their use in metabolic studies involving multiple samples. Furthermore, in cell extracts large concentrations of nucleotides and other UV absorbing components may be present which interfere with the desired analyses. Therefore, these compounds either must be removed prior to the chromatographic analysis or have retention times which do not coincide with those of the nucleosides and bases. In addition, for routine work in studies of normal and abnormal metabolisms, the analytical technique must be reliable, rapid, quantitative, and sensitive and require a minimum of sample preparation.

While ion-exchange chromatography has been the method of choice for the separation of nucleic acid components, limitations have been encountered using the totally porous or pellicular anion or cation exchangers for nucleoside and base separations. The development of microparticle chemically bonded reversed-phase

* Abbreviations: Ade = adenine; Ado = adenosine, Gua = guanine; Guo = guanosine; Hyp = hypoxanthine; Ino = inosine; Thym = thymine; Ura = uracil; Urd = uridine; Xan = xanthine; Xao = xanthosine.

partition packings for HPLC has opened new possibilities for these analyses.

Since the nucleosides and bases do not possess the ionic phosphate groups which made possible the excellent HPLC separation of the nucleotides on microparticle ion-exchange resins, the use of the partition mode for the separation of nucleosides and bases was investigated. Various applications of the partition mode of liquid–liquid chromatography have been applied to the nucleosides and bases[21–30]. However, prior to the development of chemically bonded packings, the partition mode was difficult to use because of column instability caused by bleeding of the liquid phase from the support. Thus, packings in which the liquid stationary phase is chemically bonded to an inert support have advantages over packings previously used because the stability of the packings makes it possible to obtain reproducible separations over a period of time. Furthermore, the use of microparticle packings was investigated, since it has been predicted by theory[31–35] and shown to be true in adsorption[36] and ion-exchange chromatography[37] that column efficiency increases with decreasing particle size.

Therefore, microparticle, chemically bonded reversed-phase partition packings were investigated for the development of a rapid, sensitive, and reliable analysis for the quantitative determination of the nucleosides adenosine, guanosine, xanthosine, inosine, cytidine, uridine and thymidine concomitantly with their bases in the presence of nucleotides in plasma and cell extracts.

EXPERIMENTAL

Apparatus

A Waters Assoc. Model ALC 202 liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) with a micro UV detector with a fixed wavelength at 254 nm was used. Gradients were generated by a solvent programmer accessory.

Peak areas were electronically integrated using a Hewlett-Packard Model 3380-A integrator (Hewlett-Packard, Avondale, Pa., U.S.A.) and a Model SF-770 Spectroflow Monitor variable-wavelength detector (Schoeffel Instrument, Westwood, N.J., U.S.A.), was used to obtain absorbance ratios for peak identification.

Columns

The columns used were 4 mm × 25 cm Partisil 10-ODS (Whatman, Clifton, N.J., U.S.A.) or 4 mm × 30 cm μBondapak C_{18} (Waters Assoc.). The columns were pre-packed with 10-μm, totally porous silica particles to which octadecyl groups were bonded through a Si–O–Si bond.

Chemicals

Aqueous buffers were prepared using reagent-grade potassium dihydrogen phosphate from Mallinckrodt (St. Louis, Mo., U.S.A.) and distilled water, which was first filtered through a membrane filter (Whatman). Unless otherwise indicated, the buffers were prepared in the concentrations of 0.010 *F*, after which the pH was adjusted to 5.5 using dilute potassium hydroxide.

Standards of the nucleosides adenosine, guanosine, inosine, xanthosine, thymidine, uridine, and cytidine, and the bases adenine, guanine, hypoxanthine, xanthine, thymine, uracil, and cytosine were obtained from Sigma (St. Louis, Mo.,

U.S.A.). Standard solutions were prepared at a concentration of 1 m*M* in a solution of 0.010 *F* KH_2PO_4. Before analysis, all cell extracts were filtered using a membrane filter, pore size GS, from Millipore (Bedford, Mass., U.S.A.).

Extraction procedure

For the cell extracts under Applications, the extraction procedure described by Khym[38] was used. The proteineous material was precipitated by the addition of two parts of cold trichloroacetic acid (TCA) (6% by weight). The solution was centrifuged and then filtered. To 1 ml of the supernatant fluid 1 ml of a solution of 0.5 *F* tri-N-octylamine in Freon was added. After vortexing and centrifuging the solution, the aqueous solution which was the top layer was withdrawn and stored at —4°.

Recovery experiments were carried out by adding known amounts of thymidine to a plasma matrix. These solutions were extracted according to the procedure outlined above, and per cent recovery was calculated to average 97%.

Peak identification

With cell extracts, initial peak identification was made on the basis of retention times. Standard solutions were run daily before and after the samples to monitor reproducibility of retention times. The standard addition method was also used to determine peak identities. A known quantity of a standard compound was added to the cell extract and a quantitative increase in the area of a specified peak was taken as further identification of the peak.

Some peaks in the chromatograms of cell extracts were identified by the enzymic peak-shift technique. Xanthine oxidase was used to characterize the peak with the retention time of xanthine and hypoxanthine in cell extracts. This enzyme catalyzes specifically the conversion of xanthine and hypoxanthine to uric acid.

$$\text{Hypoxanthine} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{xanthine} + H_2O_2$$

$$\text{Xanthine} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{uric acid} + H_2O_2$$

Thus both the xanthine and the hypoxanthine peak can be quantitatively moved in the chromatogram. A solution of standards and an erythrocyte sample were incubated with xanthine oxidase at 25° and a pH of 9.2 for 5 min. The reaction of the enzyme was stopped by the addition of TCA, which was subsequently removed by extraction with water-saturated diethyl ether.

Ratios of peak areas at various wavelengths were also employed to aid in identification. In such instances, triplicate runs were made at each wavelength used and the ratios of the unknown peaks in a cell extract were correlated to those of known compounds.

RESULTS

Optimal conditions

Optimal resolution of the seven nucleosides and their bases was achieved using

gradient elution, with 0.010 *F* KH_2PO_4, pH 5.5 as a low-strength eluent and a solution of methanol–water (80:20) as a high-concentration eluent. The slope of the linear gradient was from 0% of the methanol mixture to 25% over a period of 30 min and the flow-rate was 1.0 ml/min. The column temperature was ambient.

The separation of the seven nucleosides (in order of elution) cytidine, uridine, xanthosine, inosine, guanosine, thymidine, and adenosine is shown in Fig. 1A and that of the bases of these nucleosides in Fig. 1B. Under the conditions used the bases hypoxanthine, xanthine, and guanine are not completely resolved. A chromatogram of standard solutions of both the nucleosides and the bases is shown in Fig. 1C.

In most cell extracts the nucleotides will be present in substantially higher concentrations than the nucleosides and bases. Thus, either the nucleotides must be removed prior to the HPLC analysis, or it is important to be able to separate the bases

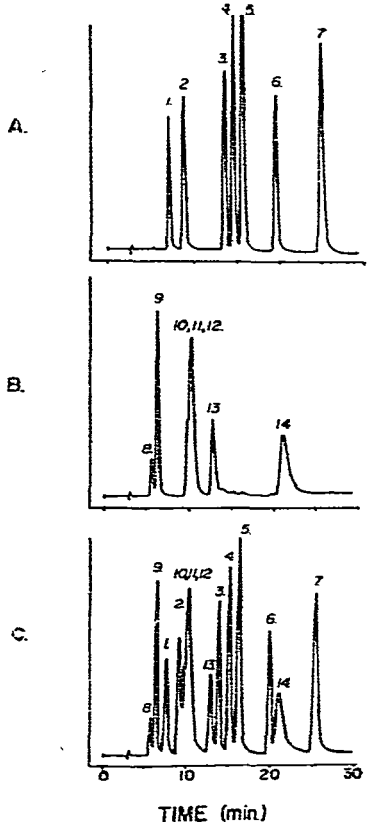

Fig. 1. (A) Separation of 5 nmoles each of the seven nucleosides cytidine (1), uridine (2), xanthosine (3), inosine (4), guanosine (5), thymidine (6), and adenosine (7). Column, μBondapak C_{18}; temperature, ambient; detector sensitivity, 0.02 a.u.f.s.; integrator setting at 254 nm, 64; eluents, (low) 0.010 *F* KH_2PO_4, pH 5.5, (high) methanol–water (80:20); gradient, linear, 0–25% of high-concentration eluent in 30 min; flow-rate, 1.5 ml/min. (B) Separation of 5 nmoles each of the corresponding bases under the same conditions as under (A). 8 = Cytosine; 9 = uracil; 10 = hypoxanthine; 11 = guanine; 12 = xanthine; 13 = thymine; 14 = adenine. (C) Separation of both the nucleosides and the bases.

and nucleosides in the presence of the nucleotides. Since it is preferable to have as few steps as possible in an analysis, we chose to investigate the latter possibility. The chromatogram in Fig. 2 shows the separation of 5 nmoles each of the seven nucleosides and bases in the presence of approximately 64 nmoles total of the mono-, di- and triphosphates of the seven nucleosides. The numbering of the peaks is the same as is used in Fig. 1C. As can be seen from the chromatogram, the nucleotides (peaks not numbered) were not retained on the column. For the nucleosides and bases, the capacity factors (k') were high enough for good separation from the nucleotides.

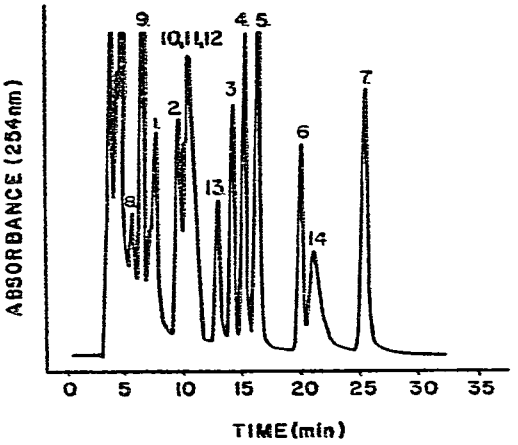

Fig. 2. Separation of 5 nmoles each of the nucleosides and bases in the presence of 64 nmoles of mono-, di-, and triphosphate nucleotides. All conditions are the same as in Fig. 1.

Reproducibility

The retention times and peak shapes were reproducible, and remained constant for sample sizes up to 100 nmoles. Variations in retention times over a month's time averaged 1.5%.

The reproducibility of peak areas is directly related to the precision of the injection and to the integration of the peak areas. Using standard solutions and syringe injection, the average variation in peak areas was 2%.

Sensitivity

Fig. 3 shows the detection of approximately 80 pmoles of each of the seven nucleosides. The lower limit of detection for the purine nucleosides, which have a higher molar absorptivity than the pyrimidines at 254 nm, was approximately 50 pmoles. The limit depends upon three parameters, *viz.* the sharpness of the peaks, the molar absorptivity of the compound of interest, and the noise of the system.

Efficiency

Because gradient elution is used for the separations, it is not possible to calculate the number of theoretical plates. However, because the time of analysis was

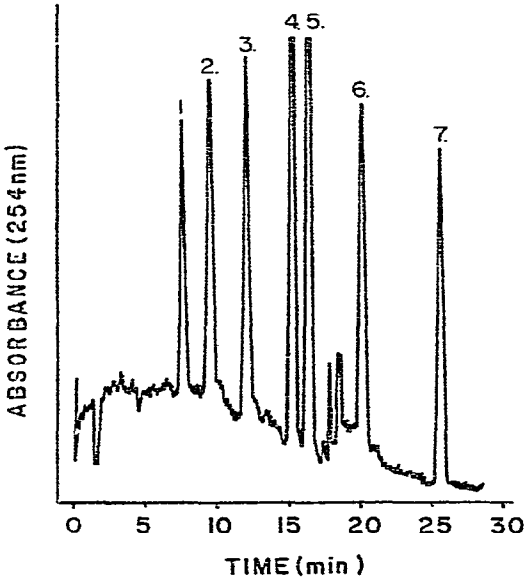

Fig. 3. Detection of approximately 80 pmoles each of the seven nucleosides. Detector sensitivity, 0.02 a.u.f.s.; integrator setting, 1. All conditions as in Fig. 1, but with the low-strength eluent at pH 5.8. Peak identification follows that in Fig. 1.

greatly shortened and the peaks decreased in width, a significant increase in efficiency was obtained over the same separation on totally porous conventional ion-exchange resins[17].

Methanol concentration

The effect of methanol concentration in the mobile phase upon the retention volumes $1/V_{R'}$ of the nucleosides and bases was investigated. Fig. 4 shows graphically the relationship between $1/V_{R'}$ and the molar concentration of methanol for the three nucleosides adenosine, guanosine, and xanthosine, and for the bases adenine and guanine. Each data point represents duplicate runs, with a precision of 2% or better. The retention volumes were obtained by multiplying the flow-rate by the retention times.

Ionic strength

In order to study the effect of ionic strength upon the separation of the nucleosides and bases, the ionic strength of the mobile phase was altered by the addition of known amounts of KCl. The pH of the mobile phase was held constant with 0.005 *F* potassium phosphate buffer. The ionic strength in a range from 0.01–0.10 Debye units had no effect upon the retention volumes of any of the nucleosides or bases. Therefore, the ionic strength of the mobile phase was not a critical factor in obtaining optimal resolution of these compounds within this range.

pH of the mobile phase

To observe the effect of pH changes, the ionic strength of the eluent was held

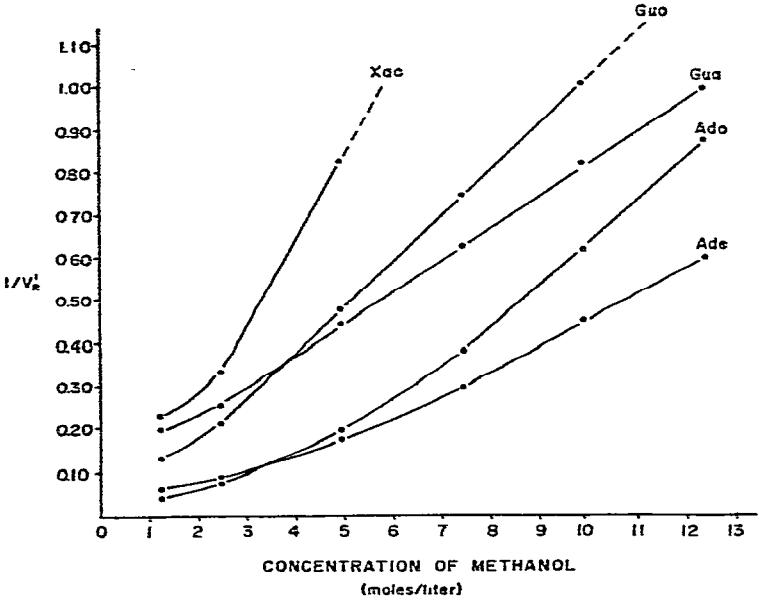

Fig. 4. Effect of increasing methanol concentration upon the retention volumes of several of the nucleosides and bases. Data were obtained under isocratic conditions. Retention volumes were calculated by multiplying the flow-rate (volumetrically measured) by the retention times.

approximately constant during isocratic elution by making all solutions 0.10 F in KCl and 0.005 F in KH_2PO_4. The pH of the system was then varied in the range of 2.8–6.8 by the addition of HCl or KOH.

The effect of pH changes on the nucleosides and bases is shown in Fig. 5. The retention volumes can be correlated with the pK_a and pK_b values of the solute molecules. There was little or no effect of pH on the bases uracil, xanthine, and hypoxanthine or on the nucleosides uridine, inosine and guanosine, whose pK_a or pK_b values are either above or below the pH range of the eluent.

Xanthosine, however, with a pK_a of 5.7, showed a marked decrease in retention volume in this pH region. At a pH of 6.8, xanthosine was eluted essentially in the void volume. However, it is interesting to note that the retention volume of xanthine did not change with pH. This is the only nucleoside–base pair studied in which the effect of pH on the retention volume of the base was significantly different from the effect of pH on the nucleoside. With adenosine and adenine, which have pK_b values of 3.50 and 4.15, respectively, the retention times were increased with increasing pH up to pH 5.8 although the slope of the rise was greater with adenosine than with adenine.

Applications

The reversed-phase partition mode was used in the HPLC analysis of nucleosides and bases in three biochemical studies. In a study of three samples of a substance which was isolated from a marine invertebrate extract, initial UV and thin-layer chromatographic data had indicated that the compound was thymine. However, it was suspected that impurities might be present.

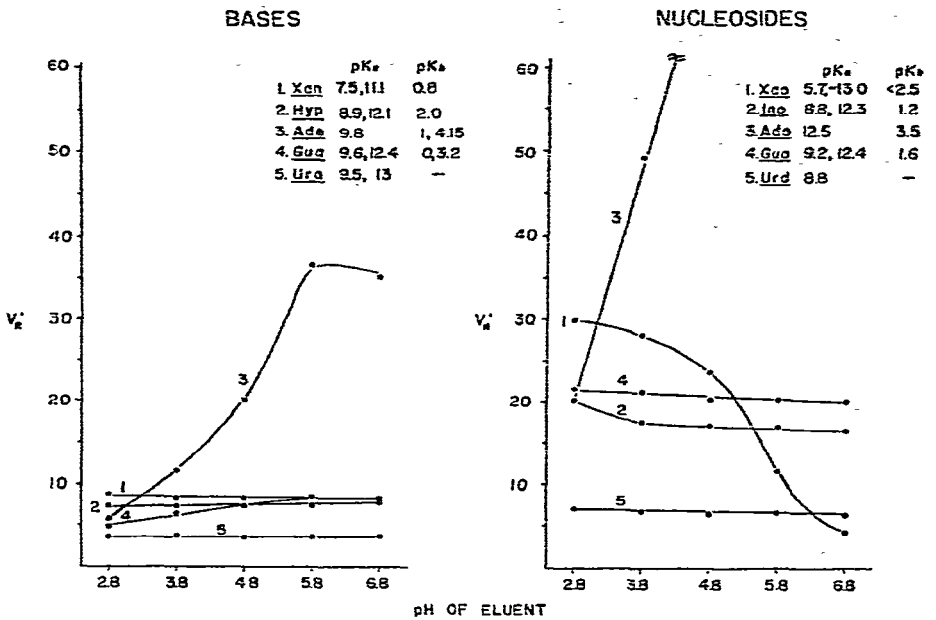

Fig. 5. Effect of pH changes on the nucleosides and bases. The data were obtained as in Fig. 4. The ionic strength of the mobile phase was held constant by making all eluents 0.10 *F* in KCl.

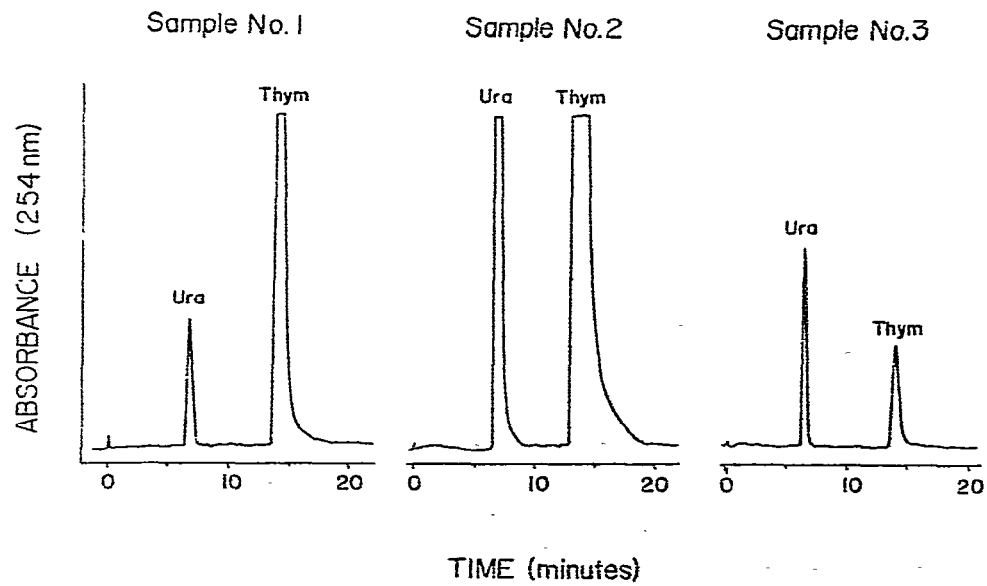

Fig. 6. Separation of components in marine invertebrate extract under the same chromatographic conditions as in Fig. 1. Instrument attenuation, 0.02 a.u.f.s.; integrator settings, 8, 16, and 32 for samples 1, 2, and 3, respectively; injection volume, 5 μl.

Using the HPLC conditions described in Methods, it was found that the samples actually contained two compounds, one with the retention time of thymine, and the other with the retention time of uracil (Fig. 6). The percentage of uracil varied from 2 to 38%.

In another study, the effect of the drug tubercidin upon the free nucleoside levels in the plasma of dogs (D-4) was observed. In the plasma of a control dog, one of the peaks present had the retention time of thymidine (Fig. 7). After treatment of the dog with tubercidin for three weeks, this peak was not present in the plasma although all the other peaks were unchanged. The total amount of thymidine contained under the peak in the control was 0.5 nmoles.

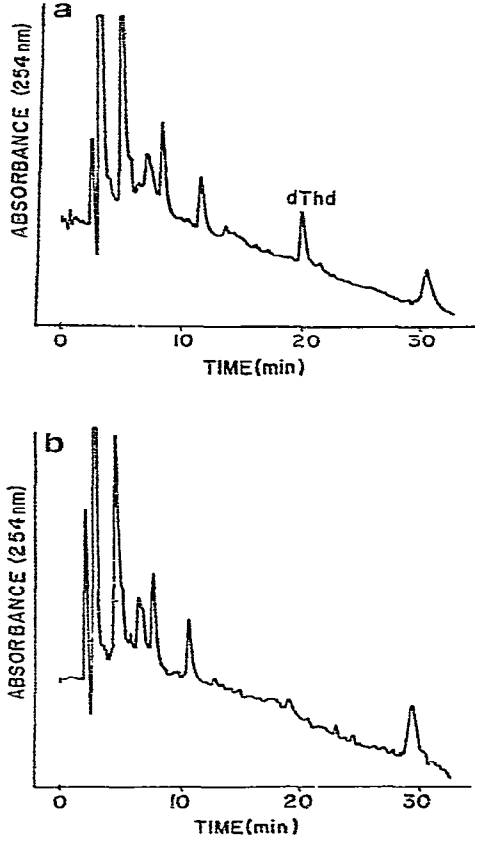

Fig. 7. Concentration of thymidine in the plasma of a control dog (D-4) (a) before and (b) after treating the dog for three weeks with the drug tubercidin. Injection volume, 25 μl; instrument attenuation, 0.02 a.u.f.s.; integrator setting, 4. All other conditions are the same as in Fig. 1.

In a third study, the levels of free nucleosides and bases from an extract of the erythrocytes of dogs (D-4) were investigated. It was found that one base or group of bases was present in significant quantities (Fig. 8a). This peak at 11 min had a retention time similar to that of guanine, hypoxanthine, or xanthine. In order to identify the

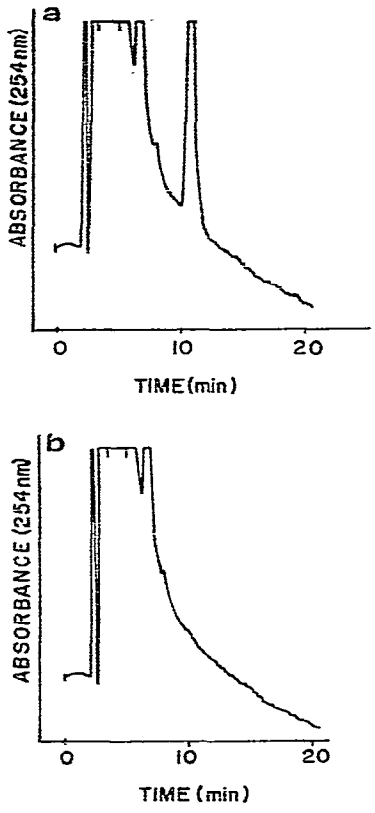

Fig. 8. (a) Detection of a peak tentatively identified as being either xanthine, hypoxanthine, or guanine. Injection volume, 25 μl; instrument attenuation, 0.02 a.u.f.s.; integrator setting, 4. All other conditions as in Fig. 1. (b) Separation of the same extract after incubation with the enzyme xanthine oxidase.

peak of interest, the enzymatic peak shift technique was used. An aliquot of the cell extract was incubated with xanthine oxidase, an enzyme which catalyzes the conversion of xanthine or hypoxanthine to uric acid. As may be seen in Fig. 8b, the peak at 11 min totally disappeared after incubation with xanthine oxidase, providing evidence that the peak was not guanine, but hypoxanthine or xanthine. To determine whether the peak was hypoxanthine or xanthine, the ratios of the peak areas at different wavelengths were determined. Using a variable-wavelength detector the area ratios (in triplicate) of standard solutions of hypoxanthine and xanthine were obtained at 260/230 nm and 250/230 nm. As shown in Table I, the ratio of the peak areas at 260/230 nm and 250/230 nm of the unknown peak was similar to that of hypoxanthine, and dissimilar to that of xanthine; thus evidence is obtained that the identity of the extract peak is that of hypoxanthine.

TABLE I

IDENTIFICATION OF THE UNKNOWN PEAK IN FIG. 8 USING AREA RATIOS OBTAINED AT 260/230 nm AND 250/230 nm

The area ratios of the unknown peaks match closely those of an authentic sample of hypoxanthine. Ratios were obtained by injecting samples in triplicate with a Schoeffel SF770 variable-wavelength detector. All other conditions as in Fig. 1.

Nucleoside	*Area ratio*	
	260 nm/230 nm	*250 nm/230 nm*
Xanthine	2.65 ± 0.04	1.98 ± 0.02
Hypoxanthine	0.67 ± 0.05	1.14 ± 0.03
Sample D-4 (1 h incubation)	0.65 ± 0.01	1.20 ± 0.07

DISCUSSION

The reversed-phase partition mode for the separation of nucleosides and bases offers several advantages over ion-exchange techniques used previously in HPLC. The majority of the naturally occurring nucleosides can be analyzed simultaneously with the bases in the presence of nucleotides in 30 min. Quantitative analyses were achieved at ambient temperatures with high sensitivity, selectivity, and efficiency. In addition, the eluents were very dilute salts and methanol which can be readily removed after fraction collections. The chromatograms were highly reproducible and column stability was good. In our laboratory columns have been used continuously for six months with no loss in efficiency.

As with all microparticle columns, proper care is essential to long life. Particulate matter must be removed from samples and solvents by membrane filtration. In addition, mechanical shock and extremes of pH and temperature must be avoided. The recommended pH range for silica-based packings is from 2 to 7.5. Due to the lyophobic nature of the stationary phase, trace organics in the solvents, as well as high-molecular-weight organics in the samples, tend to accumulate on the columns over a period of time. Unless the column is periodically flushed with a moderately polar eluent such as ethanol or acetonitrile, ghost peaks and a rising baseline during gradient elution may be encountered.

The enzymatic peak shift technique is a sensitive and accurate means of peak identification for certain nucleosides and bases. Either the disappearance of a particular peak, or the appearance of a newly formed peak, or both, may be used as a selective method of peak identification. In addition, interfering peaks may be removed by this technique.

With a variable-wavelength detector, absorbance ratios at different wavelengths can be used as a method for peak identification. A combination of absorbance ratios and enzymatic peak shift offers a selective technique for the identification of peaks in cell extracts.

Proper adjustment of pH of the samples is necessary for reproducible retention times for xanthosine. If a cell extract is very acidic, the retention times of xanthosine will vary (Fig. 5) due to small pH changes *in situ* within the column. Xanthosine is the only compound we found to be affected by small variations in pH.

Finally, reversed-phase columns from different manufacturers will produce

small differences in selectivity and retention times. For example, we found that columns from one manufacturer gave enhanced separation of xanthine–hypoxanthine–guanine, but decreased resolution of thymidine–adenine, whereas similar columns from another manufacturer gave the opposite results.

Therefore, graphs in which retention volumes are plotted against concentrations of methanol and pH are helpful for the rapid optimization of conditions for the particular column and the compounds of interest.

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