Journal of Chromatography, 121 (1976) 251–262

CHROM. 9036

ISOCRATIC SEPARATION OF SOME PURINE NUCLEOTIDE, NUCLEOSIDE, AND BASE METABOLITES FROM BIOLOGICAL EXTRACTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received December 10th, 1975)

SUMMARY

Separation of ATP, ADP, AMP, adenine, adenosine, cAMP, ITP, IDP, IMP, hypoxanthine, inosine, cIMP, the guanine series, NAD, NADPH, xanthine, 3-methylxanthine, theobromine, theophylline, and caffeine was accomplished using high-performance liquid chromatography with a microparticulate reversed-phase column. Under isocratic conditions all compounds could be eluted with reasonable resolution and retention time. Quantitation by peak height for several of the compounds was used to the 10-ng level.

INTRODUCTION

Analytical high-performance liquid chromatographic (HPLC) methods abound for separating nucleotides[1–6], nucleosides[7,8] and bases[6–9] (Fig. 1). They have been based on anion- and cation-exchange[10] and ligand-exchange[11] techniques using both isocratic and gradient elution. Separation of a mixture of the three classes[10,11] is difficult and reproducibility is often limited in the gradient mode by the care taken in running the gradient. Sensitivity is sometimes limited in gradient mode due to baseline changes with buffer concentrations often due to impurities in trace quantities, which requires an extensive and tedious purification of the buffer salts[1]. In spite of these limitations, much metabolism and cell biosynthesis work has been done using HPLC techniques usually with microgram quantities of individual components.

Methods also exist to analyze these classes by vapor phase chromatography[12–15] (nmole sensitivity), and combined gas chromatography–mass spectrometry[16–18] (pmole sensitivity). A disadvantage of the vapor phase methods is that samples must be derivatized before analysis to increase volatility and stability. Further, samples require some preliminary clean-up before derivatization, in particular the removal of salts which inhibit these reactions.

As part of another project, it was desired to identify and quantitate metabolites of ATP from the perfused cat spleen. A rapid, sensitive method was developed which would separate most of the possible metabolites under simple isocratic conditions utilizing minimal sample preparation.

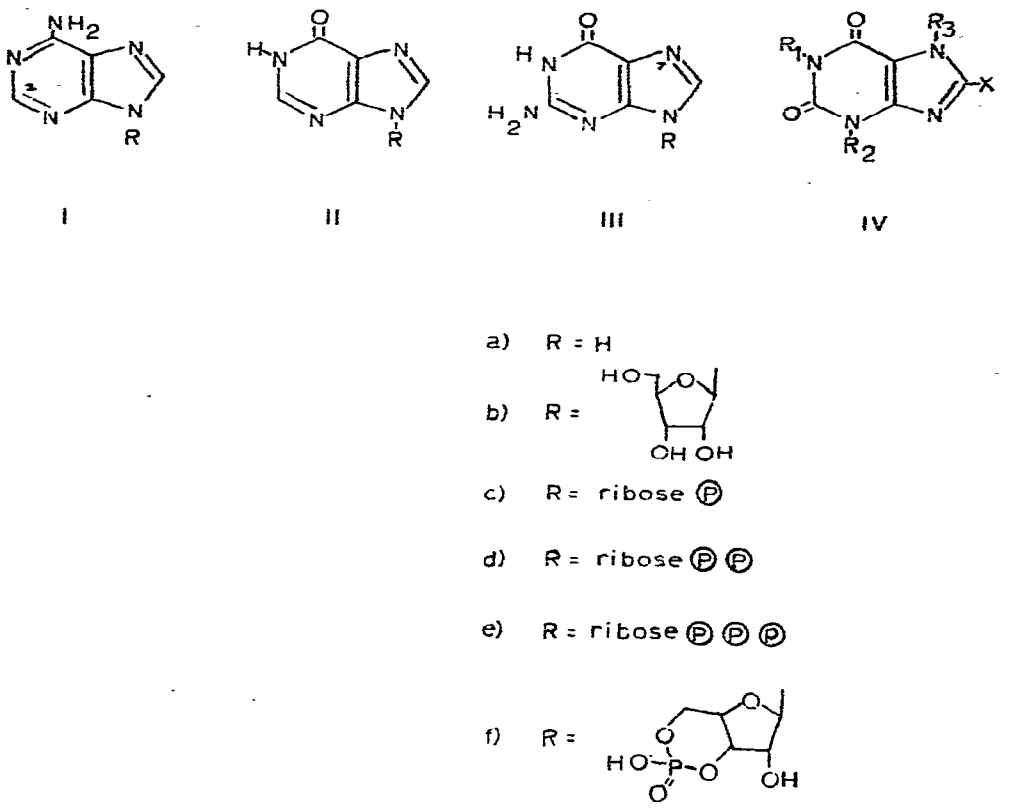

Fig. 1. Parent general structures for the adenine (I), inosine (II), guanine (III) and the xanthine (IV) compounds. Some of the abbreviations are herein listed with the structure of the various compounds determined by a combination of a parent structure plus a substituent. All nucleotides and nucleosides are 5′ and the cyclic nucleotides are 3′,5′. ATP, Ie; ADP, Id; AMP, Ic; ADE (adenine), Ia; ADO (adenosine), Ib; 2-MeADE (2-methyladenine), 2-methyl Ia; cAMP, If; ITP, IIe; IDP, IId; IMP, IIc; HYP (hypoxanthine), IIa; INO (inosine), IIb; cIMP, IIf; GTP, IIIe; GDP, IIId; GMP, IIIc; GUA (guanine), IIIa; GUO (guanosine), IIIb; 7-MeGUA, (7-methylguanine), 7-methyl IIIa; URIC (uric acid), IV, where $R_1 = R_2 = R_3 = H$, $X = OH$; XAN (xanthine), IV, where $R_1 = R_2 = R_3 = X = H$; 3-MeXAN (3-methylxanthine), IV, where $R_1 = R_3 = X = H$, $R_2 = CH_3$; THB (theobromine), IV, where $R_1 = X = H$, $R_2 = R_3 = CH_3$; THP (theophylline), IV, where $R_3 = X = H$, $R_1 = R_2 = CH_3$; CAF (caffeine), IV, where $X = H$, $R_1 = R_2 = R_3 = CH_3$.

EXPERIMENTAL

Apparatus

The HPLC system was made from components comprising a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 solvent delivery system with a U6K variable volume injector. A Laboratory Data Control (Riviera Beach, Fla, U.S.A.) dual-beam 8-μl UV flow cell (254 nm) was connected to the column for detection of UV absorbing eluents. The μBondapak C_{18} reversed-phase column (300 × 4 mm) was obtained from Waters Assoc. A pre-column (2 × 100 mm) dry packed with Waters Assoc. 40-μm C_{18} reversed-phase packing was used only to protect the column and had little effect on separations.

Chemicals

Certified ACS ammonium dihydrogen phosphate (Fisher, Fair Lawn, N.J., U.S.A.), reagent ammonium hydroxide (J. T. Baker, Phillipsburg, N.J., U.S.A.), theophylline, theobromine, caffeine, and 3-methylxanthine from K & K (Plainview, N.Y., U.S.A.), and other reagents from Sigma (St. Louis, Mo., U.S.A.) were used without further purification. cIMP was made from cAMP by heating at 60° for 2 h with aqueous nitrous acid. Inosine and hypoxanthine were prepared in the same fashion and chromatography was the same as with commercial compounds. All phosphate buffers were filtered through Whatman GF/C disks and otherwise used as obtained. Nucleotide and nucleoside standards (1–100 ng/μl) were made in glass-distilled water, stored on ice and remade every few days. Base standards were made in dilute hydrochloric acid and were remade when they discoloured although no change in chromatography was detectable.

Column care

Since a phosphate buffer system was used, care was taken to limit bacterial growth which destroys column efficiency as well as creating high column backpressure. When not in use, the column was stored in methanol or 0.05% sodium azide was added to the buffer system.

Biological samples

Samples of 45 ml of the cat spleen perfusate (Krebs buffer) were collected on ice and centrifuged at 4° for 5 min at 10,000 *g* to sediment red blood cells and platelets. The decanted solutions were stored frozen at −78° until the time of analysis. They were then thawed with 45 ml absolute ethanol. After rotary evaporation to dryness at 30°, the salt mixture was suspended in 2 ml of water and centrifuged to remove excess salts. Aliquots (10 μl) were injected directly into the high-pressure liquid chromatograph for analysis. Fractions were collected (from the 300–400 μl preparative injections) and radioactivity measured by liquid scintillation techniques.

RESULTS

Separation of adenine related compounds

ATP, ADP and AMP were eluted within 6 min from the micro C_{18} column using a 0.05 *M* ammonium dihydrogen phosphate buffer at pH 6.0 with a flow-rate of 2 ml/min as shown in Fig. 2. In general, the elution of the nucleotides was insensitive to ionic strength, but was sensitive to changes in pH. Elution was accelerated by the addition of methanol as indicated in Table I. Isocratic separation of all the adenine containing compounds studied was possible; however, as can be seen in Table I, adenine, adenosine, and cyclic adenosine monophosphate exhibited rather long retention times. If a step gradient was instituted after the elution of AMP in which the same buffer system was made to 5% methanol, the elution shown in Fig. 3 could be obtained. This simple solvent switching enabled the resolution of all adenine containing ATP metabolites in a reasonable time.

Separation of the inosine and guanine series

The elution of the inosine containing molecules is shown in Fig. 4. The step

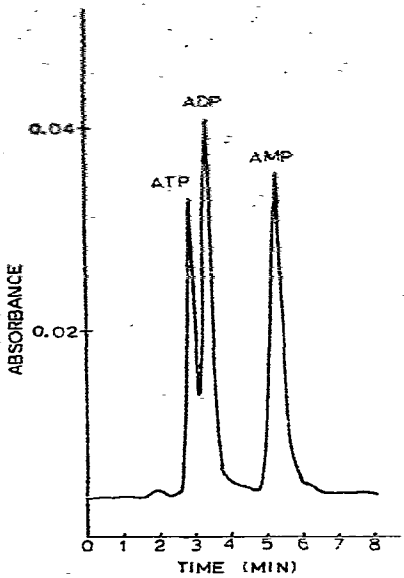

Fig. 2. Separation of ATP, ADP, and AMP (*ca.* 300 ng each in 3 μl). The column void volume is approx. 2.2 ml. Operating conditions: 0.05 *M* $NH_4H_2PO_4$ buffer at pH 6.0, a flow-rate of 2 ml/min, an operating pressure of approx. 2000 p.s.i. and room temperature.

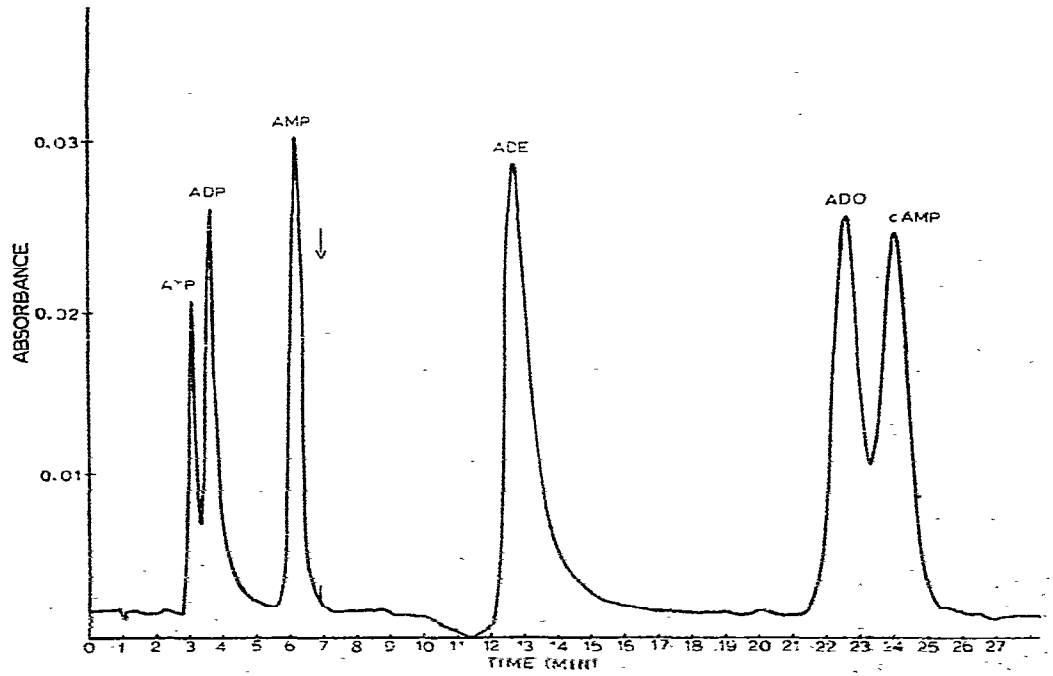

Fig. 3. Separation of ATP, ADP, AMP (*ca.* 200 ng each in 10 μl total), and adenine, adenosine and cAMP (*ca.* 500 ng each in 10 μl total). Operating conditions were the same as in Fig. 2. The step gradient from phosphate buffer to phosphate with 5% methanol was initiated at the arrow after AMP eluted.

TABLE I

RETENTION DATA OF PURINE NUCLEOTIDES, NUCLEOSIDES AND BASES UNDER DIFFERENT ISOCRATIC CONDITIONS

A: Average retention time (min) of 9 values with standard deviation of 3% determined with 0.05 $NH_4H_2PO_4$ (pH 6.0) and 2 ml/min flow-rate. B: Average of 3 retention times determined with the same conditions as in A but with different column behavior. C: Relative retention parameters determined with three sets of retention times with two different columns. D: Relative retention parameters obtained using the phosphate buffer with 2% methanol (v/v). The NADPH retention time is 7.52. E: Relative retention parameters obtained using the phosphate buffer with 5% methanol. The NADPH retention time is 4.54.

*Compound**	*A*	*B*	*C*	*D*	*E*
ATP	3.08	3.27	0.75	0.66	0.49
ADP	3.69	3.83	0.71	0.60	0.40
AMP	6.24	6.24	0.52	0.39	0.16
ADE	14.2	13.3	−0.08	−0.44	−0.82
ADO	36.4	38.1	−1.93	−1.78	−2.28
2-MeADE					−2.19
cAMP	38.8		−2.10	−2.05	−2.53
ITP	1.97		0.84		
IDP	2.22		0.82		
IMP	3.14		0.75		
HYP	4.96	5.17	0.62		
INO	10.40	10.7	0.17		
cIMP	12.57		0.00		
GTP	2.00	2.10	0.84	0.72	0.63
GDP	2.22	2.27	0.82	0.72	0.60
GMP	3.12	3.11	0.76	0.65	0.52
GUA		5.34	0.59	0.41	0.22
GUO		12.57	0.03	−0.14	−0.24
7-MeGUA		13.9	−0.08	−0.10	
NAD	9.08		0.28		
NADPH	12.53	12.9	0.00	0.00	0.00
URIC	2.83	3.10	0.77		0.47
XAN	5.68	5.85	0.55		0.14
3-MeXAN				−0.49	−0.83

* For abbreviations see legend of Fig. 1.

gradient was not necessary due to the reduced affinity of the inosine containing molecules to the reversed-phase column. Complete resolution of these components from the adenine counterparts shown in Fig. 3 was not possible in particular within the first 3 min. The relative retention times, however, were sufficiently unique to allow unambiguous assignment in all but a few cases; for example, IMP, GMP and ATP co-elute as well as GTP and ITP, GDP and IDP.

Separation of the xanthine bases

Uric acid, hypoxanthine, xanthine could also be separated under isocratic conditions as can be seen in Fig. 5. The cofactors NAD and NADPH were co-injected with these compounds as well as adenine to demonstrate their elution characteristics.

The isocratic separation of caffeine and its xanthine metabolites theophylline and theobromine as well as 3-methylxanthine and xanthine is shown in Fig. 6. The

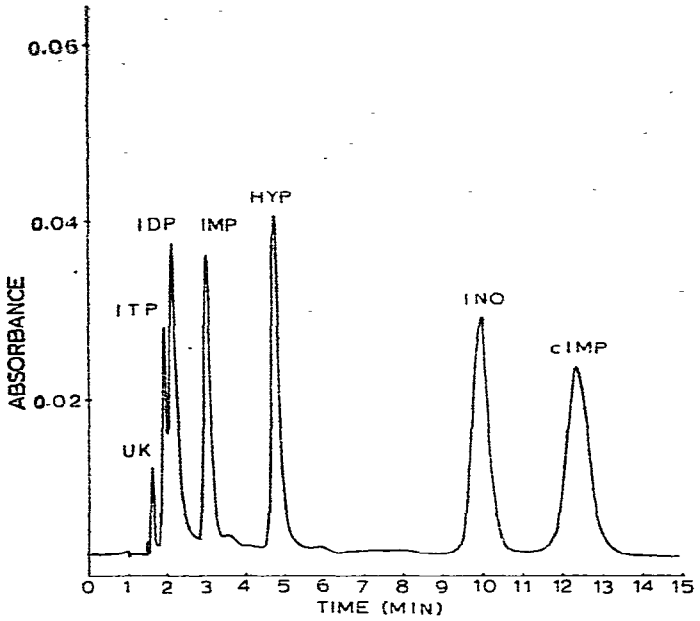

Fig. 4. Separation of ITP, IDP, IMP, hypoxanthine, inosine and cIMP (*ca.* 200 ng each in 10 μl) in a completely isocratic mode. Operating conditions were the same as in Fig. 2. UK is not identified.

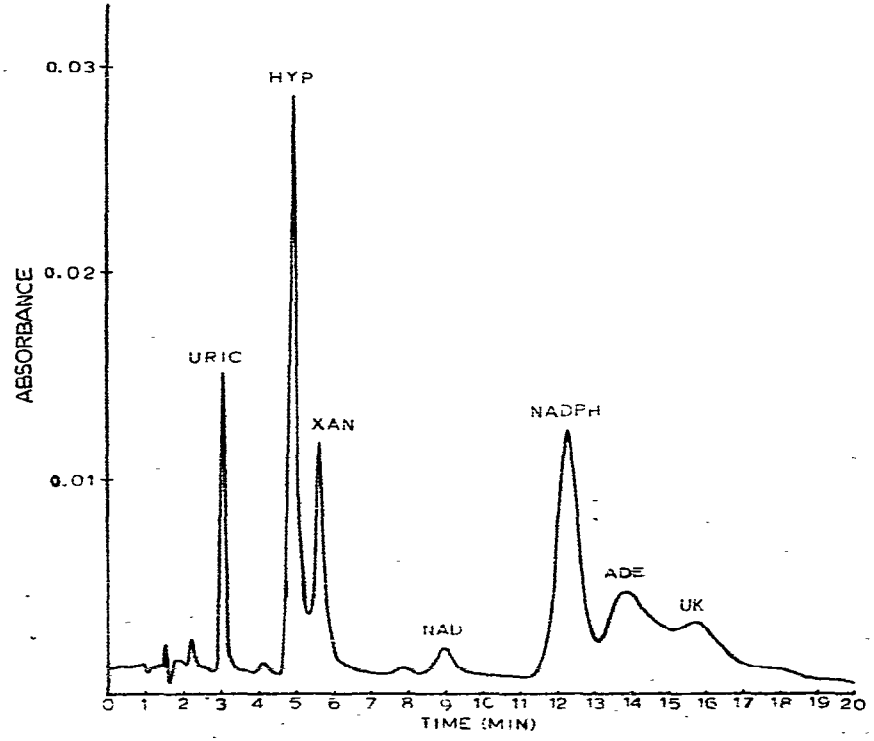

Fig. 5. Separation of uric acid, hypoxanthine, xanthine, and adenine (*ca.* 150 ng each in 10 μl total) and NAD plus its unidentified decomposition product (UK) and NADPH (*ca.* 500 ng each). Operating conditions were the same as in Fig. 2.

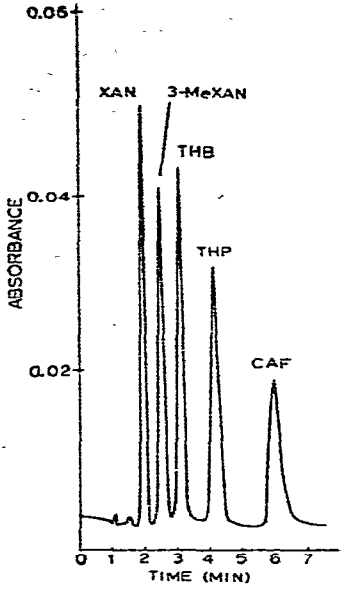

Fig. 6. Separation of xanthine, 3-methylxanthine, theobromine, theophylline, and caffeine (*ca.* 200 ng each in 10 μl). Operating conditions: 0.05 M $NH_4H_2PO_4$ buffer at pH 6.0 which was made to 25% methanol, flow-rate of 2 ml/min and an operating pressure of approx. 2500 p.s.i.

buffer system contained 25% methanol which reduced the retention of caffeine but still allowed complete resolution of the other xanthines. Good HPLC conditions for these compounds are obtained with 15–30% methanol either in the phosphate buffer or water at a flow-rate of 2–3 ml/min. Both systems perform the same. With 15% methanol at 2 ml/min the analysis is complete in 12.5 min and with 25% methanol in 6 min. At 3 ml/min baseline resolution is maintained and the analysis time is 4 min. It was found that the elution of the non-phosphated compounds was the same in the presence or absence of the phosphate buffer. Thus the same separation as shown in Fig. 6 and for uric acid, hypoxanthine, xanthine, and adenine in Fig. 5 was possible substituting water for the 0.05 ammonium dihydrogen phosphate buffer.

Reproducibility

Several factors make retention time an unsatisfactory parameter for compound identification. Notwithstanding the 3% standard deviation in retention time, column characteristics change to a much greater extent with usage, and sometimes suddenly of no apparent cause. Cleaning the column with water followed by methanol or tetrahydrofuran sometimes restores previous characteristics. Three different columns were used throughout this study and the absolute retention time for hypoxanthine varied as much as 15% when columns were new. A relative retention parameter is defined which was found to be rather insensitive to these changes in column behavior unlike the absolute retention times. The parameter is defined as:

$$\frac{t_{R_{std}} - t_{R_x}}{t_{R_{std}}}$$

where t_{R_x} is the absolute retention time of compound x and $t_{R_{std}}$ is the absolute retention time of the standard compound. NADPH (Fig. 5) was chosen as an arbitrary standard due to its unique and intermediate retention time. Retention parameters were not constant if the solvent system was changed from the aqueous phosphate buffer to a solvent with methanol or if the flow-rate was altered more than 20%. They do, however, change in a predictable fashion. If the retention parameters are plotted against the change in operating condition, the curves for most of the compounds are parallel. Reported also are retention parameters determined with two different methanol concentrations. With these values and the retention time of NADPH supplied in the legend of Table I, retention times of the other compounds may be determined.

Quantitation

The pharmacologic study which employed this chromatographic technique required only rough quantitation; therefore a complete study of the applicability of this technique of quantitative analysis of the compounds was not done. The simple

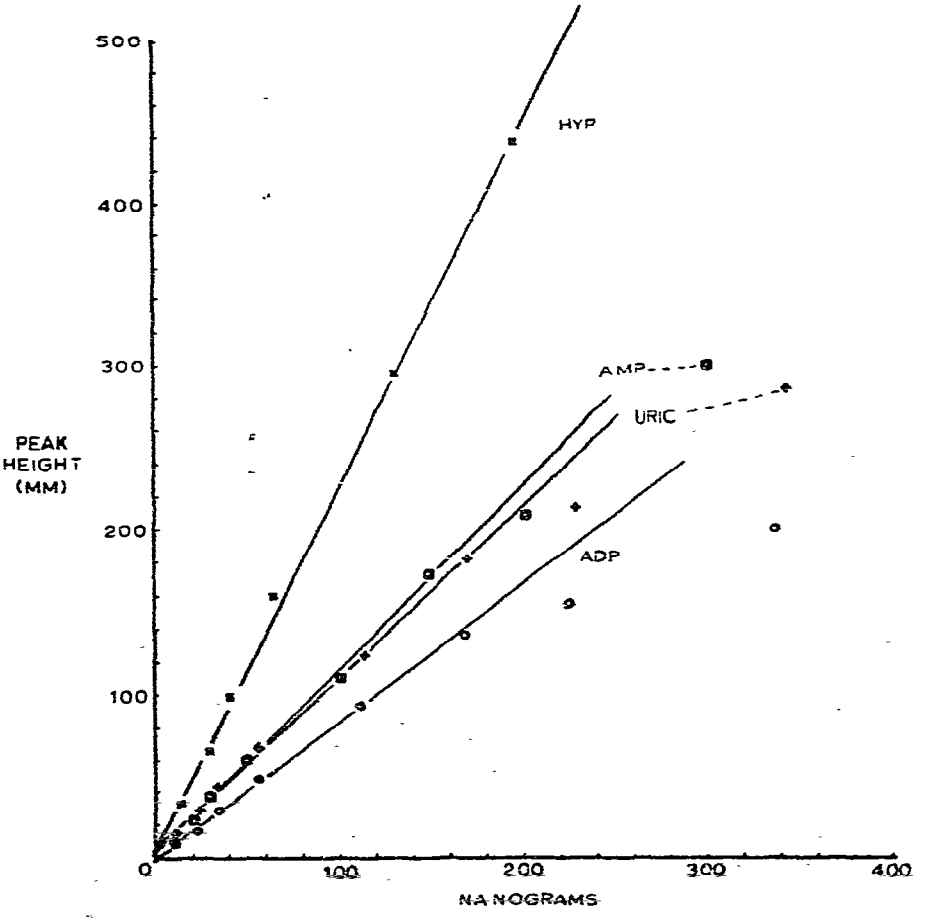

Fig. 7. Calibration curves of peak height *vs.* quantities injected of hypoxanthine, AMP, uric acid and ADP. Operating conditions were the same as in Fig. 2.

method of peak height measurement relative to volume injected was found satisfactory. Fig. 7 illustrates the set of standard curves for uric acid, hypoxanthine, AMP, and ADP. These curves were generated by injecting 5–150 μl of a precisely known standard solution containing 2–3 ng/μl of each component. Linearity was lost around 200 ng due to peak broadening with the large volume injected. The standard deviation for individual injections is close to 10% below 25 ng and at higher levels approaches 3%. The most probable source of error is in the injection technique using a 1-ml syringe for greater than 25 μl.

Application to a biological mixture

Fig. 8 shows a preparative profile of the perfusate of a cat spleen following injection of [^{14}C]ATP. The major component eluting between 5 and 6 min contained 70% of the radioactivity and was tentatively identified as hypoxanthine both by retention time and co-injection of authentic hypoxanthine. The tentatively identified component was preparatively isolated on the reversed-phase column using distilled water as the eluent. This effectively removed the buffer salts and allowed trimethylsilyl derivatization. Identity was confirmed as hypoxanthine by gas chromatography–mass spectrometry. The identity of AMP and adenine in the perfusate has been established only by co-elution with standards.

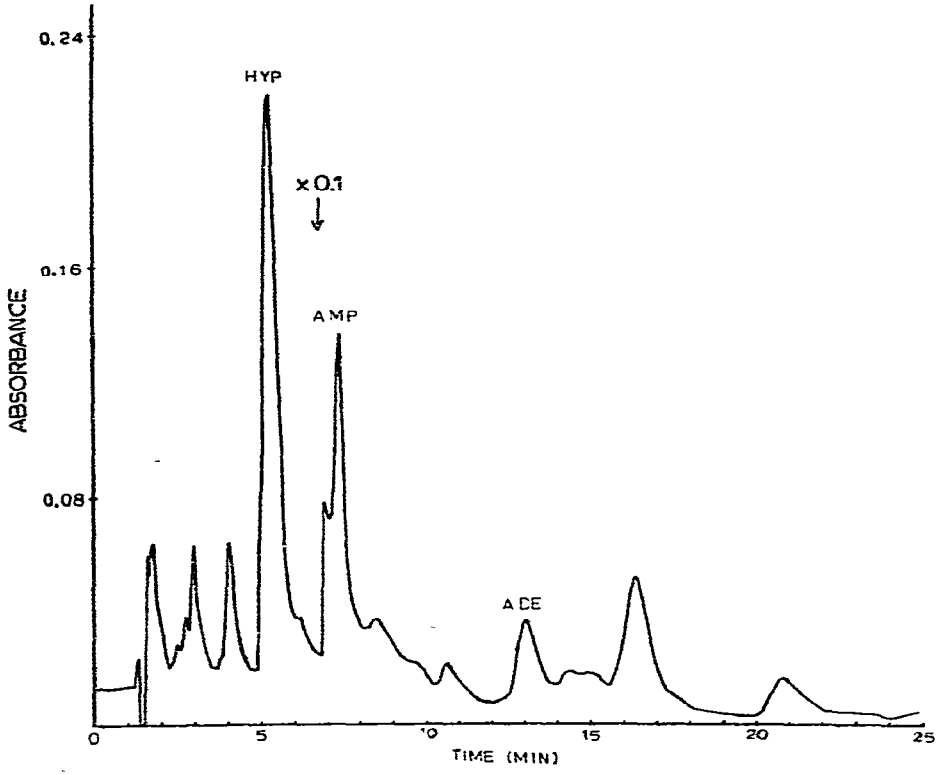

Fig. 8. Separation of components from a spleen perfusion (300 μl). Full-scale absorbance was 0.32 O.D. prior to 7 min at which time it was changed to 0.032 O.D. to detect minor components. Operating conditions were the same as in Fig. 2.

DISCUSSION

In reversed-phase chromatography, compounds are separated on the basis of differential phase distribution or partition between the two phases, the hydrophobic column packing and the hydrophilic solvent. Compounds will have particular affinities for the non-polar hydrocarbon phase of the column and also for the mobile phase. Molecules adhere to the stationary phase by virtue of their non-polar components in contrast to ion exchange methods where adherence is due to ionized functions. Elution characteristics can be changed (1) by changing the compound's affinity for the stationary phase by making it more or less hydrophobic, *e.g.* state of ionization of the compound or (2) by altering affinity for the solvent, *e.g.* ionic strength or polarity changes in the solvent. Decreasing polarity of an aqueous solvent with methanol or acetonitrile displaces the molecules from the stationary phase by competition or mass action effects[19,20].

Considering specific separations (Fig. 1), the nucleotides offer a straightforward example. Since column affinity is fixed by the adenine moiety, and mobile phase affinity increases with added phosphate moieties, the elution order in the adenine series is ATP, ADP, and then AMP. This also holds for the guanine and inosine nucleotides. Similar arguments apply for AMP and adenosine, the polar ionized phosphate enhances mobile phase affinity and AMP elutes first. Somewhat puzzling is the relative elution order of adenosine and adenine. Looking at the change in retention time observed by removing the phosphate from AMP, it might be predicted that removing the ribose from adenosine would greatly increase the column affinity of adenine, thus giving adenine a very long retention time. As can be seen in Table I, this is not observed; the polarity of adenine (or solvent affinity) is greater than for adenosine as it elutes more quickly. The ribose does not appear to greatly increase solvent affinity; but of possible importance is the free N(9)H group of adenine.

Methyl substitution can play a significant role in the reversed-phase chromatography of these purines; 7-methylguanine has the methyl group in the position analogous to the N(9) position of adenine and 7-methylguanine has a retention time longer than that of guanosine while that of guanine itself is much shorter. 2-Methyladenine eluted much later than adenine and slightly after adenosine which is at least qualitatively the same as the guanine and 7-methylguanine case. In these two cases, the effect of methyl substitution appears to change elution characteristics to the same

TABLE II

RETENTION TIMES OF XANTHINE RELATED BASES

A: Retention times with 0.05 *M* $NH_4H_2PO_4$ (pH 6.0) with 15% methanol of the final volume added after the pH had been adjusted. B: Retention times with 25% methanol in the buffer as in A. For abbreviations of compounds see legend of Fig. 1.

Compound	*A*	*B*
XAN	2.20	1.83
3-MeXAN	3.46	2.50
THB	4.87	3.10
THP	7.16	4.15
CAF	12.47	5.97

degree, independent of methyl position. However, isomeric methylxanthines have enough difference in retention characteristics to allow separation (Table II) as discussed below.

Another example which shows the importance of the overall polarity of the molecule is the comparison between adenosine and cAMP which elute in the same region. At pH 6.0 cAMP (pK_a 7.2) is predominantly in its less polar, protonated form. This combined with the cyclic structure obviously minimizes polarity of the phosphate. Indeed, cAMP elutes after adenosine.

There are some general features worth noting in the relative elution orders for various compounds. Comparing adenine to hypoxanthine, it is seen that the lactam moiety (the most stable form for hypoxanthine)[21] greatly increases solvent affinity relative to an aromatic amine moiety. Comparing guanine to hypoxanthine it is found that addition of the primary amine to the existing lactam structure plays only a slight role relative to hydrogen, on the resultant retention time.

Another series of interest is hypoxanthine, xanthine, and uric acid (pK_a 8.8, 7.7 and 5.75, respectively). Based only upon the number of polar functions or pK_a, the predicted elution order would be: uric acid, xanthine, and hypoxanthine. However, in the experimental result xanthine elutes after hypoxanthine. The literature has been active in the area of tautomerism of the purines[21,22]. Molecular orbital calculations indicate that the preferred form for both xanthine and hypoxanthine is the lactam (keto) configuration. Pullman and Pullman have pointed out that these compounds are predominantly in the lactam form "although the importance of the lactim form in some circumstances may be far from negligible"[21]. The very late elution of adenine which is predominately in the aromatic tautomer suggests that aromaticity may increase column affinity relative to solvent affinity. While it is difficult to relate adenine to the xanthine series directly, the observed elution order (hypoxanthine prior to xanthine in the reversed-phase system) may be a function of the greater proportion of xanthine in the aromatic, lactim form relative to hypoxanthine. Uric acid may be more aromatic and have a smaller dipole than the othres, but it certainly has a greater solvent affinity. Uric acid is about half ionized at pH 6.0 and as such elutes quite early. At pH 3.8, uric acid elutes later (at 4.6 min) while xanthine and hypoxanthine are unchanged (Table I).

A series of interest regarding methyl group effects is xanthine to caffeine. Xanthine has the greatest solvent affinity due to the two lactam functions. Caffeine elutes last because of the replacement of three protons (decreased solvent affinity) with relatively lipophilic methyl groups. Theobromine and theophylline, each having two methyl substituents, elute before caffeine and after 3-methylxanthine which elutes after xanthine (Fig. 6). The two dimethylxanthine isomers, theobromine and theophylline, are nicely resolved due to the difference in affinities resulting from the different methyl positions.

ACKNOWLEDGEMENTS

We wish to thank Werner Mosiman for the cat spleen preparations and Dr. Norman Weiner for helpful discussions. These investigations were supported by a research grant from the National Institutes of Health (GM 20457).

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