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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR BASE AND NUCLEOSIDE ANALYSIS IN EXTRACELLULAR FLUIDS AND IN CELLS

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SUMMARY

High-performance liquid chromatography based methods for the study of the metabolism of purine and pyrimidine bases and nucleosides have been developed. These methods, using 200—50 μl samples of extracellular fluids and employing isocratic separations, can measure a wide range of compounds. Hypoxanthine, xanthine and uridine concentrations in plasma from normal men are relatively stable. Species differences have been detected: concentrations of cytidine are higher in rat and mouse serum than in man, since the concentrations of uridine are similar; purine/pyrimidine ratios may be different. Fetal calf serum used for tissue culture contains about a 40 times higher concentration of hypoxanthine than the less-effective calf serum. Use of the methods appears to be justified in the assessment of the metabolic damage due to severe hypoxia and/or ischaemia.

INTRODUCTION

Interest in the metabolism of purines, pyrimidines, their nucleosides and nucleotides has been recently stimulated by developments in the study of inborn errors of metabolism [1] and in the biochemistry of "hypoxia". During ischaemia there is a reduction in the adenine nucleotide concentration, especially ATP, in cells (see ref. 2). Such reductions in ATP are associated with diminished erythrocyte survival [3] and renal survival [4]. However, measurement of adenine nucleotides requires tissue samples, which are difficult to obtain in serial or in clinical studies. Methods for the estimation of ATP metabolites, the purines and their nucleosides, in extracellular fluid are therefore required since there is extensive evidence [2, 4] of purine release by cells during hypoxia and by organs during ischaemia.

The isolation from biological material and subsequent identification of many

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purines, pyrimidines and their nucleosides, especially from urine [5], followed the development of ion-exchange chromatography by Cohn [6]. Despite development such methods were not suitable for physiological and pathological work, especially on small or on serial samples. There were at least two major problems; the low concentration of purines and their nucleosides in extra- and intracellular fluids, and the large number of closely interrelated and rapidly changing metabolites. The development of high-performance liquid chromatography (HLPC) with sensitive ultraviolet absorbance detectors can now provide methods of sufficient sensitivity and specificity to measure the large numbers of interrelated compounds in body fluids. The practicability of the methods allows large numbers of samples to be analysed and thus the detection of rapid changes.

Brown et al. [7] reported a method for the analysis of purine and pyrimidine bases and their nucleosides by HPLC, and Hartwick and Brown [8] further developed this type of method for the selective analysis of adenosine. This group and others showed that good separations of standard compounds could be achieved by HPLC with reversed-phase columns which have generally been prepared commercially. However, the application of these methods to biological material has been limited perhaps because of the use of gradients or the limited sensitivity even of recent methods [9].

The present paper describes the development and application of a flexible isocratic HPLC method of high sensitivity and resolution using readily available 5- and 3-μm packings and standard equipment. Purine and pyrimidine bases and their nucleosides were quantitated in a wide variety of extracellular fluids and cell samples. These methods have been proved to be suitable for serial physiological and pathological studies over a period of three years.

MATERIALS

Purine and pyrimidine bases, nucleosides and other standards were obtained from Sigma, London, Great Britain, unless otherwise stated. Solvents and other chemicals were obtained from BDH, Poole, Great Britain; AnalaR grade was used unless otherwise stated. The methanol used in HPLC mobile phases was from Rathburn Chemicals, Walkerburn, Great Britain. Allopurinol used as an internal standard was supplied by Burroughs Wellcome, London, Great Britain. For HPLC, only all-glass double-distilled water, which was stored in glass containers and checked for the absence of plasticiser peaks on HPLC, was used.

Enzymes used in microchemical identification were xanthine oxidase (E.C. 1.2.3.2) from Koch-Light Labs., Colnbrook, Great Britain, and guanase (E.C. 3.4.5.3) and purine-nucleoside phosphorylase (E.C. 2.4.2.1.) from International Enzymes, Windsor, Great Britain.

A liquid chromatograph Model ALC 200 (Waters Assoc., Hartford, Great Britain) incorporating a U6K injector, M600A pump and Model 440 absorbance detector operating at 254 and 280 nm was used for all chromatography.

METHODS

Column packing

We have used ODS-Hypersil (Shandon Southern Ltd., London, Great Britain) packing. These are silica microspheres 5—7 μm, and more recently 3 μm, in diameter with a bonded phase of octadecyl groups. Both prepacked columns (250 × 5 mm) and laboratory packed columns were employed, the latter proving to be more robust. Columns were packed by upwards displacement at $4.3 \cdot 10^3$ kPa (6000 p.s.i.) [10] using a CPIII column slurry packer (Jones Chromatography, Llanbradach, Great Britain). The packing suspension and packing solvent was isopropanol. Columns were subsequently equilibrated with 200—300 ml of 60% (v/v) methanol—water and 200—300 ml of 30% (v/v) methanol—water before use.

After flushing to remove buffer salts, columns were stored overnight in 5—20% (v/v) methanol—water containing 0.2% (w/v) sodium azide. Columns were cleaned after every 100—200 samples, or if a noticeable loss of efficiency occurred, with 60% (v/v) methanol—water, isopropanol, and aqueous 0.2% (w/v) EDTA. This, and periodic repacking of 1—3 mm of the column top, restored a column to a usable efficiency, with a theoretical plate count of about 8000—16,000 per 250 mm, as measured with hypoxanthine.

Mobile phases

All buffer solutions were filtered through a Millipore 0.5-μm filter and degassed under vacuum, using all-glass containers at each stage. Mobile phases containing organic solvent, 0.9—1.0% (v/v) methanol, gave good peak shapes and consequent quantitation as well as prolonging column life.

The pH of the mobile phase had to be chosen carefully if peaks of interest were to be separated from contaminants, which sometimes varied in extracts of the same type from different people. Changes in pH and ionic strength altered the behaviour of some contaminants more than that of purines and pyrimidines. The mobile phase for the best resolution of the components of an extract had sometimes, therefore, to be determined. Generally 0.01 mol/l potassium dihydrogen phosphate (pH 6.5) containing 1% (v/v) methanol, which gave good resolution of standard purines, pyrimidines and their nucleosides, was a starting point. Concentrations of potassium dihydrogen phosphate ranged from 0.001 to 0.05 mol/l with the pH adjusted to 3.5—6.75 with 10 mol/l sodium hdyroxide or orthophosphoric acid. For analysis of the nucleosides thymidine and adenosine somewhat higher concentrations of methanol, 5—10% (v/v), gave optimal sensitivity and resolution.

The retention of compounds on the columns could be decreased by increasing the temperature of the column and mobile phase from the usual ambient temperature of 21°C, to 55°C. Although increased retention was found at 0°C, peak shapes were poor and high viscosity of the mobile phase caused marked pressure increases. A simple water jacket for the column (Wright Scientific) coupled to a standard TU14 Tempunit (Techne) is now used and gives more reproducible retention of compounds.

Quantitation of compounds

Complex chromatograms are obtained from biological samples; thus an internal standard with a retention time close enough to compounds of interest to allow easy quantitation may be obscured by contaminating peaks. Since complex samples can take about 30 min to analyse by HPLC, it is not practicable to inject a solution of standard compounds before each sample. Moreover, retention of a compound was variable throughout a day, although such variations are reduced by controlling the column temperature, so that simple measurements of peak height were inaccurate and measurement of peak area by graphical methods impossible, due to the small width of most peaks. However, column efficiency as measured by the number of theoretical plates (N) remains constant during a day, although varying by ± 10% on column storage. For these reasons N was measured with hypoxanthine and a mobile phase of 0.01 mol/l potassium dihydrogen phosphate (pH 6.5) to which 1% (v/v) methanol was added, at 1.0 ml/min. For each compound of interest, retention and peak height were recorded. It can be shown that for any peak, concentration, C, is related to peak height, H, and retention time, t_R, as follows

$$C \propto H\, t_R / N^{1/2}$$
$$C = F\, H\, t_R / N^{1/2}$$

where F is a constant for each compound dependent on detector response and instrument settings. For each compound of interest F was measured and found to be constant (± 2%) and independent of N, retention time and, for most compounds, mobile phase. However, when peak shape changed with pH, with for example guanine, F had to be measured again. This somewhat unusual method of measurement was satisfactory.

The calculation of concentrations in cells was initially based on cell number determined by counting, or on cell protein as measured with the Folin phenol reagent. In order to obtain concentrations that were comparable with concentrations in extracellular fluids and which bore some relation to available kinetic constants of metabolising enzymes and membrane "carriers", results were expressed relative to cell volume determined from existing median values [11].

Identification of purine bases and nucleosides in extracts

The identity and purity of peaks was established by a variety of methods:

(1) Retention relative to an internal standard which remained relatively constant throughout the life of the column.

(2) Ratios of absorbance measured as peak heights at 254 and 280 nm which was a useful characteristic for many compounds [12].

(3) Peak shape, there was characteristic tailing of adenine.

(4) Response to changes in pH of the mobile phase. Cytidine, xanthine, and guanine change their retention and in the case of guanine, peak shape, with pH changes whereas hypoxanthine and uridine do not change.

(5) Treatment with the relatively specific enzyme preparations described above caused the disappearance of the appropriate substrate and appearance of its product on the chromatogram. Incubation was at 37°C, after adjusting the pH of the extract with solid Tris or 2 mol/l hydrochloric acid to an optimum for the relevant enzyme. The efficiency of enzyme treatment and duration of

incubation needed was checked with standard compounds at the appropriate concentration. When resolution from contaminants was impracticable, peaks could be measured before and after enzyme treatment and the concentration of the substrate thereby determined.

(6) Absolute concentrations of urate were high in plasma and amniotic fluid but not in cerebrospinal fluid. Urate has a high absorbance at 280 nm compared to that at 254 nm, thus providing an identifiable endogenous internal standard with a short retention time eluting before most of the purine bases of interest and uridine. The added internal standard, allopurinol, eluted after the purine bases and uridine but before inosine (Fig. 1).

(7) The addition of standard compounds to extracts sometimes showed that the retention time of an unknown was not exactly identical with that of the added standard.

The procedures commonly used were relative retention time, 254/280 nm absorbance ratio and enzyme treatment. The only measured components not identified by all the above procedures were uridine and cytidine. Their chromatographic behaviour, however, was distinctive.

Estimation of the efficiency of extraction and overall precision of analysis of hypoxanthine, xanthine and inosine from plasma

The extraction procedure for plasma, amniotic fluid, cerebrospinal fluid or blood cells was an adaptation of that of Khym [13]. Two volumes of ice-cold fresh trichloracetic acid (TCA) solution, 6—14% (w/v) depending on the type of sample analysed, were added to the fluid or cell suspension and the resulting precipitate removed by centrifugation. The supernatant was then extracted three times with 1.5 volumes of water-saturated diethyl ether to remove the TCA. The aqueous layer, now pH 4—5, was passed through a 0.5-μm Millipore filter prior to HPLC. About 0.2 ml of biological sample was adequate for this procedure although volumes of 20 μl have been extracted satisfactorily. All extracts were stored at —20°C.

The use of Tris and phosphate buffers to neutralise the TCA in the extracts was associated with the appearance of ultraviolet-absorbing compounds with similar retention times to allopurinol on ODS-Hypersil columns; these impurities could not be eliminated. Since high sensitivities were needed for purine base and nucleoside analyses, especially in cerebrospinal fluid in which concentrations were often less than 1 μmol/l, neutralisation of extracts with buffers could not be used; the longer extraction of TCA with diethyl ether was necessary.

In order to estimate the efficiency of the extraction method, a 5-ml sample of human blood plasma was incubated for 30 min with xanthine oxidase (0.5 ml, Koch-Light) to remove endogenous hypoxanthine and xanthine. The reactions were stopped by adjustment of the pH to 2.5 with concentrated hydrochloric acid. Known amounts of solutions of hypoxanthine, xanthine, both 1.3—30.1 μmol/l, and inosine 0.6—16.3 μmol/l were added to aliquots of this plasma and mixed thoroughly. Two volumes of 6% (w/v) TCA were added to the plasma and the extracts analysed at least twice [14].

Precautions in sample handling

The stability of TCA extracts was similar to that of solutions of standards. Purine base concentrations showed no definite change over periods of as long as six months. Brown and Miech [15] noted little change even in nucleotide concentrations of comparable extracts stored at −20°C for over one month.

Purine release from blood cells into plasma is detectable almost immediately after sampling [16], as suggested by earlier experimental work [17], and affects intracellular nucleotide concentrations significantly by about 40 min after sampling [18]. If consistent results were to be obtained from plasma extracts the rapid removal of blood cells by centrifugation within 15 min of sampling, with care taken to avoid cell lysis, was important; thereafter plasma was stable at −20°C. In general most samples except amnotic fluid were extracted within 48 h. In order to separate debris and contaminating cells all samples were better centrifuged. Only slight changes in the HPLC pattern were noted with samples of amniotic fluid when stored for over 24 months; these changes were not consistent. Cell preparations were routinely extracted upon receipt. The suspending medium was removed by centrifugation and aspiration, and the cellular pellet was resuspended in the appropriate volume of TCA solution.

RESULTS

The isocratic separation of standards is shown in Fig. 1, with the difficult separation of hypoxanthine from guanine in Fig. 2. Good resolution of the components of plasma and cerebrospinal fluid extracts was always possible (Figs. 3 and 4) with a mobile phase of appropriate pH and ionic strength. Sensitivity for hypoxanthine was about 1 pmol but depended on the volume injected and the concentrations of interfering compounds; this was especially important in samples from patients with renal failure.

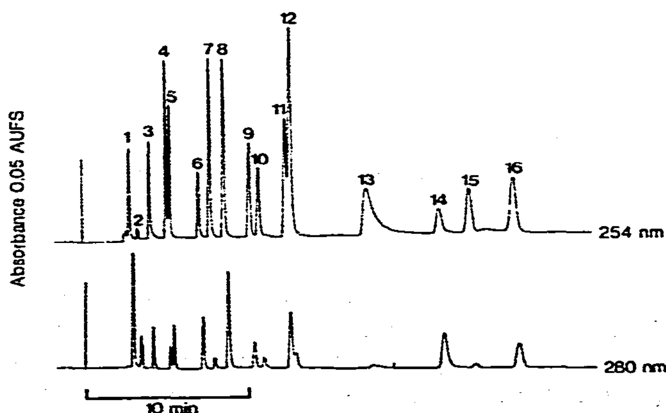

Fig. 1. Chromatogram of standard compounds (30—100 pmol). Peaks: 1 = orotic acid, 2 = uric acid, 3 = cytosine, 4 = uracil, 5 = pseudouridine, 6 = cytidine, 7 = hypoxanthine, 8 = xanthine, 9 = uridine, 10 = oxypurinol, 11 = thymine, 12 = allopurinol, 13 = adenine, 14 = 7-methylguanine, 15 = inosine, 16 = guanosine. Conditions: 25 × 0.5 cm column of Shandon C_{18} 3-µm ODS; mobile phase, 1 ml/min, 0.004 mol/l KH_2PO_4 (pH 5.8) with 1% (v/v) methanol at 30°C column temperature. N = 14,000.

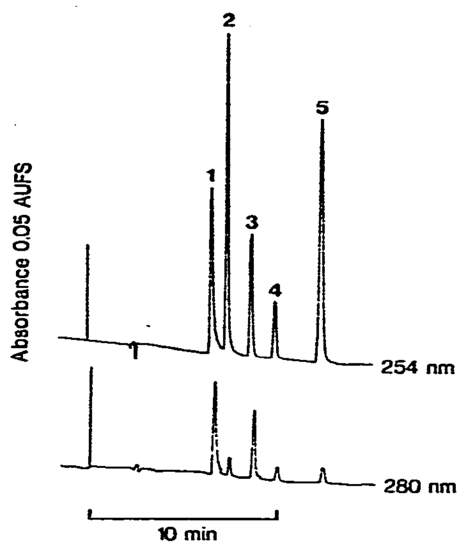

Fig. 2. Chromatogram of standard compounds (30—100 pmol) showing separation of guanine (1), from hypoxanthine (2), xanthine (3), uridine (4) and allopurinol (5). Mobile phase adjusted to pH 3.2; otherwise conditions and column performance as in Fig. 1.

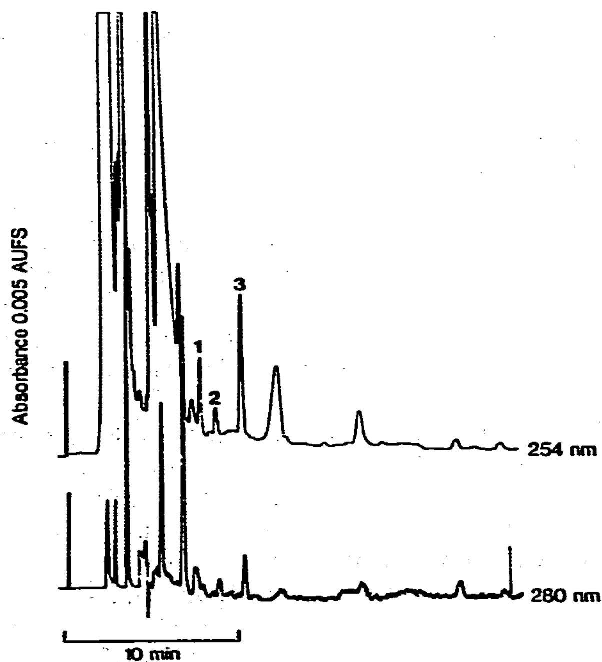

Fig. 3. Chromatogram of an extract of plasma (20 μl) using EDTA as an anticoagulant showing hypoxanthine (1), xanthine (2) and uridine (3). Conditions and column performance as in Fig. 1.

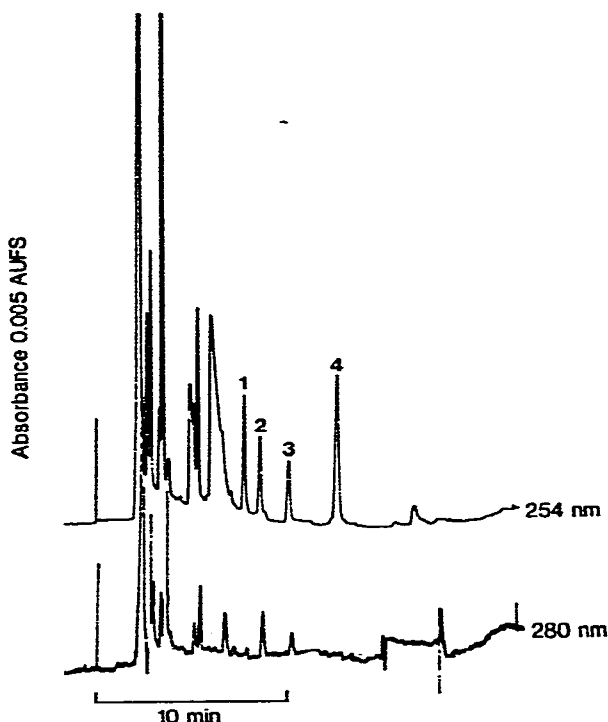

Fig. 4. Chromatogram of an extract of cerebrospinal fluid (20 μl) showing hypoxanthine (1), xanthine (2), uridine (3) and the internal standard allopurinol (4). Conditions and column performance as in Fig. 1.

Estimation of the efficiency of extraction and overall precision of analysis of hypoxanthine, xanthine and inosine from plasma

The mean recovery ± S.D. of hypoxanthine was 99.4 ± 3% (n = 12) in the concentration range 1.3—10 μmol/l and 100.3 ± 2% (n = 10) in the concentration range 10—30 μmol/l. For xanthine the mean recovery ± S.D. was 115 ± 15% (n = 18) in the concentration range 1—30 μmol/l. The presence of interfering peaks in this extract accounted for the higher and more variable values. The mean recovery ± S.D. for inosine was 82.3 ± 8% (n = 18) in the concentration range 1—16 μmol/l.

Estimates of precision obtained by repetitive analyses of single pools may be lower than those obtained during practical applications of a method. A series of samples were analysed in duplicate; using the differences between duplicate estimates [14] on a series of 29 different plasma samples the S.D. was ± 1.8 for a mean of 37.4 μmol/l, a coefficient of variation of 4.9%. For xanthine, the mean, S.D. and coefficient of variation were 3.8 ± 0.3 μmol/l, and 8.7% on 22 sets of duplicate analyses.

Using the difference between duplicate HPLC estimations on the same extract from a series of different plasma samples, the coefficients of variation for hypoxanthine were 2.4% (n = 18), 2.9% (n = 14), 1.3% (n = 14) and 1.9% (n = 12) for the concentration ranges 3—12, 20—25, 38—50 and 70—95 μmol/l, respectively. Similarly, for xanthine the coefficient of variation was 7.6% (n = 32) in the concentration range 0.1—50 μmol/l, for uridine 13.4% (n = 18) in

the concentration range 0.1—30 μmol/l and for inosine 6.0% (n = 8) in the concentration range 0.1—30 μmol/l. Using the above results for hypoxanthine, it appears that about half the error in the results may be due to the extraction and about half due to the HPLC.

Purine base, urate and uridine concentrations in extracellular fluids

The concentrations of hypoxanthine, xanthine, uridine and urate in plasma from four normal human males were obtained from serial samples of blood taken through an indwelling needle at 0, 30, 60, 90, 140 and 210 min in the middle of a working day. The concentrations showed no systematic or large differences in this "normal" working day. The results are therefore shown as mean ± S.D. (Table I); adenine, cytidine and inosine were not detected in these samples.

TABLE I

CONCENTRATIONS OF HYPOXANTHINE, XANTHINE, URATE AND URIDINE IN PLASMA FROM SERIAL SAMPLES FROM FOUR MEN

Samples from an indwelling needle were obtained at 0, 30, 60, 90, 150 and 210 min during a working day. Separation was by HPLC and detection by absorbance at 254 nm.

Subject	Concentration (μmol/l, mean ± S.D.; n = 6)			
	Hypoxanthine	Xanthine	Urate	Uridine
R	2.04 ± 0.6	0.61 ± 0.21	230 ± 30	4.37 ± 0.34
V	1.53 ± 0.14	0.68 ± 0.12	230 ± 30	4.21 ± 0.67
M	1.19 ± 0.27	0.92 ± 0.45	250 ± 30	3.04 ± 0.52
D	1.92 ± 0.75	0.85 ± 0.34	240 ± 50	3.35 ± 0.55

TABLE II

PURINE, CYTIDINE AND URIDINE CONCENTRATIONS IN FETAL CALF, CALF, RAT AND MOUSE SERA

Samples were from separate batches of material for tissue culture. The numbers in parentheses are separate batches in which the compound was detected. Analysis was by HPLC with detection at 254 nm.

	Concentration (μmol/l; mean ± S.D.)				
	Hypoxanthine	Xanthine	Uridine	Urate	Cytidine
Fetal calf	74.7 ± 31.9 (9)	92.0 ± 28.9 (9)	5.1 ± 2.1 (6)	130 ± 80 (7)	ND*
Calf	1.8 ± 1.9 (3)	0.3	3.8 ± 2.0 (3)	13 ± 9 (3)	1.3
Rat	0.7 ± 0.3 (9)	0.4 ± 0.5 (4)	3.9 ± 2.3 (9)	75 ± 40 (8)	9.7 ± 3.3 (9)
Mouse	1.1 ± 0.8 (3)	0.8 ± 0.5 (3)	5.0 ± 3.1 (3)	6.1 ± 1.4 (3)	3.6 ± 2.6 (3)

*ND = not detectable, i.e. a concentration of less than about 0.1 μmol/l.

Cytidine was found in rat and mouse sera (Table II) as well as in more limited samples from pig, rabbit, guinea pig, chicken, rhesus monkey, dog and sheep. Uridine as well as hypoxanthine and xanthine concentrations have been comparable in all species so far studied although the concentrations of hypoxanthine were low in rat plasma. The very low concentration (< 0.1 μmol/l) of cytidine in human plasma was confirmed using ultrafiltrates prepared with a filter with an exclusion limit of 25,000 (Amicon, Woking, Great Britain).

TABLE III

PLASMA AND CELLULAR CONCENTRATIONS OF PURINES, THEIR NUCLEOSIDES AND URIDINE IN BLOOD FROM FOUR NORMAL MEN

Plasma and erythrocytes were prepared by centrifugation, lymphocytes and polymorphonuclear neutrophil leucocytes (PMN) by a dextran—Ficoll based method; compounds were separated by HPLC and estimated from their absorbance at 254 nm.

	Concentration (μmol per l of plasma or cell volume; mean ± S.E.M.)					
	Hypoxanthine	Guanine	Xanthine	Uridine	Inosine	Guanosine
Plasma	1.5 ± 0.2	0*	0.46 ± 0.09	3.2 ± 0.6	0	0
Erythrocytes	11.0 ± 5.8	0	0	1.5 ± 0.3	0	0
Lymphocytes	139 ± 22	84 ± 21	0.2 ± 0.1	35 ± 7	64 ± 10	0
PMN	375 ± 98	71 ± 1.8	9.7 ± 5.5	63 ± 9.9	80 ± 13	20 ± 8

*0 indicates a concentration of less than about 0.1 μmol/l.

The most striking finding on analysing sera used for tissue culture purposes is the variable but approximately 40-fold higher concentrations of hypoxanthine and xanthine in fetal calf sera (Table II).

Cellular concentrations of hypoxanthine, guanine, xanthine, uridine, inosine and guanosine

The erythrocytes, polymorphonuclear neutrophil leucocytes and lymphocytes from human blood are frequently studied after standard separation methods (see ref. 19). The major purine base and nucleoside contents of erythrocytes and leucocytes with related plasma samples from four normal men are shown in Table III. Uridine concentrations in plasma are higher than in erythrocytes whereas hypoxanthine concentrations are higher in erythrocytes.

The pattern of purine bases has shown differences between different types of cells separated by standard methods; in lymphocytes the guanine concentration can be relatively high [20] whereas in polymorphonuclear neutrophil leucocytes xanthine may be relatively high. The relatively high rates of protein synthesis in lymphocytes and of oxidative and peroxidative metabolism in polymorphonuclear neutrophil leucocytes are probably related to these patterns. Overall concentrations would probably be lower in vivo because some nucleotide breakdown is to be expected during the standard separation methods used to produce lymphocyte and especially polymorphonuclear neutrophil leucocyte preparations.

Chromatography of the regulatory nucleotide A2'p5'A2'p5'A on ODS-Hypersil

A variety of nucleotides can be chromatographed on reversed-phase columns using our system. The most novel of these is the nucleotide A2'p5'A2'p5'A, the core of a variety of phosphorylated derivatives from interferon-treated cells [21]. The core material, which was supplied by Dr. I.M. Kerr (NIMR), had a retention time relative to adenosine of 0.81; similar relative times were 0.56 for A2'p5'A and 0.51 for adenine. Good separations and adequate retention was achieved with a mobile phase of 0.004 mol/l potassium dihydrogen phosphate with 10% (v/v) methanol (pH 6.5) at 1 ml/min. Extracts from a variety of tissues contained no major components with a similar retention time to the core nucleotide. Estimation of these compounds by the selective removal of

phosphate groups and estimation of the core nucleotides in tissue extracts may therefore be possible if sensitivity is adequate.

DISCUSSION

Clinical studies of blood plasma using xanthine oxidase and an oxygen electrode by Saugstad [22] have shown raised concentrations of oxypurines after hypoxia. However, this method failed to detect any oxypurines in cerebrospinal fluid from 15 of 39 patients [23]. The present HPLC method has been sensitive enough to estimate hypoxanthine in more than 100 cerebrospinal fluid samples.

The use of thin-layer chromatography (TLC) and densitometry [24] allowed the detection of raised concentrations of hypoxanthine in newborn urine after hypoxia but could not detect any increase in xanthine. A spectrophotometric method detected an increase in xanthine but not in hypoxanthine [25]. However, using the HPLC method we have shown an increase in both hypoxanthine and xanthine concentration of amniotic fluid, derived from fetal urine [2] and in urine from hypoxic newborn (unpublished). The identification of adenine in human plasma using TLC [26], despite the failure of other workers to detect this compound, suggests that results of purine analysis using TLC should only be accepted with caution.

The recent availability of better packing materials for HPLC has allowed much greater sensitivity and resolution than those previously demonstrated with isocratic systems [27]. This was needed for inosine concentrations in plasma. Even a recent HPLC method [9] for hypoxanthine required two isocratic runs and 500 μl of plasma (cf. Fig. 3).

For repetitive analysis of extracellular fluids for those compounds, hypoxanthine and uridine, that are exchanged between cells [28], isocratic conditions are more practicable than gradients [29, 30]. We found that a gradient was required only for separation of adenosine. However, due to co-chromatographing impurities plasma extracts required further purification before estimation of adenosine by HPLC. A single gradient separation of one TCA extract was impracticable.

It was necessary to select a suitable batch of reversed-phase material for the separations which were needed. The most difficult relevant separation was hypoxanthine from guanine (Fig. 2); this was achieved with resolution to the baseline with the initial batch of ODS-Hypersil. However, some later batches, despite giving a more consistent performance, better peak shapes and efficiencies, did not resolve these two compounds. Less trimethylsilylation or "capping" of the C_{18}-coated packing material allowed this separation to be made but not others (for example, xanthine and uridine) that are also biochemically important. There was associated with less trimethylsilylation a marked tailing of the peaks. It was generally possible with a selection of columns and mobile phases to achieve the necessary separation in a biochemical sample because not all the bases were always present. For example, guanine was not present in extracellular fluids in amounts comparable to hypoxanthine.

The use of external purines by cells is well established [28]. High concentrations of hypoxanthine in fetal calf serum would reduce the energy cost of cell

growth in culture [31] and avoid the need for the long pathway of de novo purine synthesis. Hypoxanthine may therefore be one of the factors responsible for the capacity of fetal calf serum to establish fibroblast cultures from human cell samples [32].

The results in Table II suggest that there may be differences between human, rat and mouse cells in the concentrations of extracellular purines relative to pyrimidines to which these cells are adapted. Experimentally large imbalances of purines relative to pyrimidines are capable of causing metabolic upset [33].

The applications of the present series of methods to physiological and pathological problems in man [2, 16, 34] and pig [35] has provided evidence for their reliability. The relative constancy of the results in plasma (Table I) suggest that physiological and pathological changes could be detected relatively easily.

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