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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOSIDES AND BASES IN HUMAN PLASMA

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SUMMARY

A reversed-phase high-performance liquid chromatographic (HPLC) method has been developed for the estimation of purines, pyrimidines and their congeners in biological fluids. An initial HPLC separation allowed the collection of a number of effluent fractions, each of which contained a single component of interest. The re-application of these fractions to a second HPLC separation permitted the resolution and quantification of nanogram amounts of these components. Isocratic elution with volatile buffers renders the samples amenable to automatic sampling procedures or lyophilisation. Data are presented on the application of the method to the analysis of nucleosides and bases in human plasma.

INTRODUCTION

Purine and pyrimidine antimetabolites such as 6-mercaptopurine, allopurinol and 5-fluorouracil have been in clinical use for many years. More recently, interest has grown in the use of naturally occurring nucleosides and bases to modulate the effects of anticancer agents [1—13]. We have been pursuing the reversal of methotrexate toxicity with nucleic acid precursors, both in animals and man [1—4].

For these studies a method was required which would allow us to monitor a number of purines and pyrimidines (hypoxanthine, Hx; thymine, T; oxypurinol, Op; allopurinol, Ap; and thymidine, TdR) in the plasma of patients. Methods available during the early stages of our studies were prohibitive in terms of sensitivity and the time involved in quantitation of single components [14—16]. Several methods have been published for the estimation of nucleosides or bases

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using high-performance liquid chromatography (HPLC) [17—29]. Unfortunately none of these was suitable for our purposes. The majority allowed only the estimation of one or two components, or combinations present in relatively high concentration in the samples used. The method described here was developed to allow us to monitor the particular nucleosides and bases of interest in single, relatively small plasma samples. With minor modifications the method has proved suitable for the estimation of a wide range of naturally occurring purines and pyrimidines and their congeners.

Selective absorption has proved valuable in certain cases where a class of related compounds has been separated from unwanted components. For example Gehrke and co-workers [22,24,27] used a boronate column to separate ribonucleosides from urine; Brown and Bye [19] used a Cu^{2+} Chelex-100 column to separate purine bases from plasma and urine. These methods could not be adapted to our purposes, since we were interested in deoxyribonucleosides and bases which are not retained on a boronate column. Further we wished to estimate both purines and pyrimidines. Group separations of nucleosides and bases on polyacrylamide gel columns as described by Khym [30] and used by Jackson et al. [6] were lengthy and complex and thus unsuitable for our purposes.

An initial separation system was evolved which allowed the collection of a number of effluent fractions each of which contained a single component of interest together with a limited number of contaminants. An isocratic reversed-phase HPLC system was used for the initial separation as this gave optimal resolution of IR and TdR from the large compound peak eluting between 17 and 20 min (Fig. 1b and c) under these conditions. It was found that different reversed-phase packings exhibited different retention characteristics for complex standard mixtures. Columns which were totally unsuitable for the initial separation were found to be extremely useful in the separation of components which co-eluted with the peaks of interest in the initial plasma run. Application of the fractions collected from the initial separation to an appropriate second chromatographic separation permitted the resolution and quantification of the peaks of interest. Isocratic elution conditions were again used for the second chromatographic separation as this facilitated the use of automatic sampling procedures without the problems of washing/re-equilibration or loss of sensitivity due to baseline fluctuations associated with gradient elution.

EXPERIMENTAL

Apparatus

Chromatographic studies were conducted with Waters Assoc. (Milford, MA, U.S.A.) equipment comprising two Model 6000A solvent delivery systems, controlled by a Model 660 solvent programmer; a two-channel (254 nm and 280 nm) Model 440 absorbance detector or Model 450 variable-wavelength detector. A Model U6K universal injector was used for initial plasma runs and a Waters intelligent sample processor (WISP) Model 710 was used for the application of fractions eluted from these runs. Absorbance changes were monitored using a Rikadenki Model B381L three-pen recorder. Columns were thermostatically

maintained at 23.5°C using a Haake model FE constant temperature circulating bath connected to a column temperature control block (Waters Assoc.).

Columns used for this study were 10-μm μBondapak C-18 (Waters Assoc.), 10-μm Lichrosorb RP-18 (Merck, Darmstadt, G.F.R.) and 5-μm Zorbax C-8 (Dupont, London, Great Britain). LiChrosorb RP-18 and μBondapak C-18 columns were packed by a method similar to that of Broquaire [31]. A Magnus P6000 slurry packing unit connected to a Micromeritics 705 slurry reservoir was used. A stirred slurry of packing material in methanol—7.4 m*M* sodium acetate (80:20) was forced upwards into the column by pumping methanol—7.4 m*M* sodium acetate (50:50) into the reservoir.

Chemicals

Nucleosides and bases used in these investigations were obtained from Sigma (London, Great Britain) with the exception of allopurinol (Burroughs Wellcome, London, Great Britain) and 4,6-dihydroxypyrazole (3,4-D)-pyrimidine (oxypurinol) (Aldrich Milwaukee, WI, U.S.A.). Other chemicals of analytical grade were purchased from the following sources: methanol (James Burroughs, London, Great Britain); perchloric acid and potassium hydrogen carbonate (Hopkin & Williams, Chadwell Heath, Great Britain); acetic acid (Koch-Light, Colnbrook, Great Britain) and ammonia (Fisons, Loughborough, Great Britain).

Buffers

All buffers were prepared fresh daily using water from a Milli-QZD 20 230 00 reagent-grade water system (Millipore, Bedford, MA, U.S.A.). Water and buffers were filtered through a HA 0.45-μm filter (Millipore) and methanol through a 0.5-μm Fluoropore filter before use.

Sample preparation

Fresh heparinised blood samples were centrifuged at 600 *g* for 10 min. Plasma was collected on ice and 0.5 vol of 1 *N* perchloric acid added. The samples were first centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was then subjected to a 10,000 *g* spin for a further 10 min at 4°C. This second centrifugation removed a fine haze which was apparent in many of the patients' plasma samples. The supernatants were then neutralised with solid potassium bicarbonate, and after standing on ice for 15 min, the potassium perchlorate precipitate was removed by filtration through a Millex 0.22-μm filter (Millipore).

Chromatography of deproteinised plasma samples

Initial separations were performed using a 300 mm × 4 mm I.D. μBondapak C-18 reversed-phase column (Waters Assoc.) running isocratically in 0.025 *M* ammonium acetate pH 5.0 at a flow-rate of 2 ml/min (Fig. 1). Injection volumes of 500 μl of deproteinised plasma were used, and appropriate fractions collected into sample bottles for the WISP and stored in batches for further analysis. Sample collection was determined by the elution profiles of appropriate standards injected onto the column immediately prior to the plasma run (Fig. 1a). Following the collection of the final fraction a 5-min gradient to 100% methanol was initiated. This served to wash lipophilic plasma

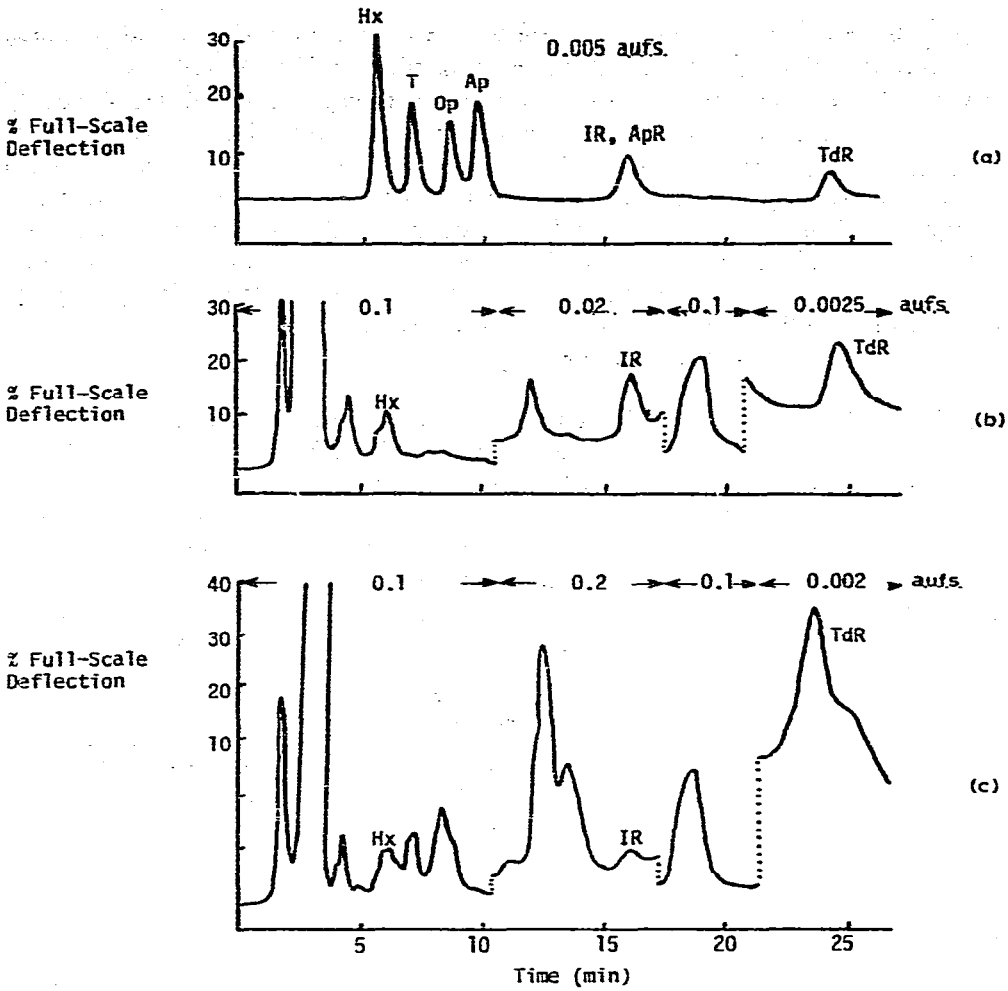

Fig. 1. Chromatograms obtained from the injection of 500-µl samples of (a) standards, (b) healthy human plasma extract, (c) patients' plasma extract onto a 300 mm × 4 mm I.D. µBondapak C-18 reversed-phase column eluted isocratically with 0.05 *M* ammonium acetate pH 5.0. Flow-rate 2 ml/min; temperature 23.5°C; detection 254 nm; a.u.f.s. as indicated between arrows. Peaks: HX, hypoxanthine; T, thymine; Op, oxypurinol; Ap, allopurinol; IR, inosine; ApR, allopurinol riboside; TdR, thymidine.

components from the column. Following the elution of these components a reverse gradient to buffer was selected and the column allowed to equilibrate before the application of further samples.

Analytical procedure

Fractions collected from the initial plasma runs were thawed and shaken to ensure homogeneity and placed in the carousel of the WISP together with a standard bottle in position 1. The WISP was then programmed to inject appropriate volumes of each sample together with reference injections of the standard after every five samples. The recorder was routinely used to monitor absorption at 254 nm at sensitivity settings of 0.02, 0.005 and 0.001 absorption units full

scale (a.u.f.s.) allowing quantitation of a wide range of nucleoside or base concentrations in a single automated run.

Hypoxanthine analysis

For the quantitation of hypoxanthine a 300 mm × 4 mm I.D. LiChrosorb 10-μm RP-18 column was used in an isocratic system of 0.025 *M* ammonium acetate pH 5.0 with a flow-rate of 2 ml/min (Fig. 2). Sample volumes of 100 μl were used with a run time of 7 min which led to a total automated run time of about 8 h for the analysis of 40 samples.

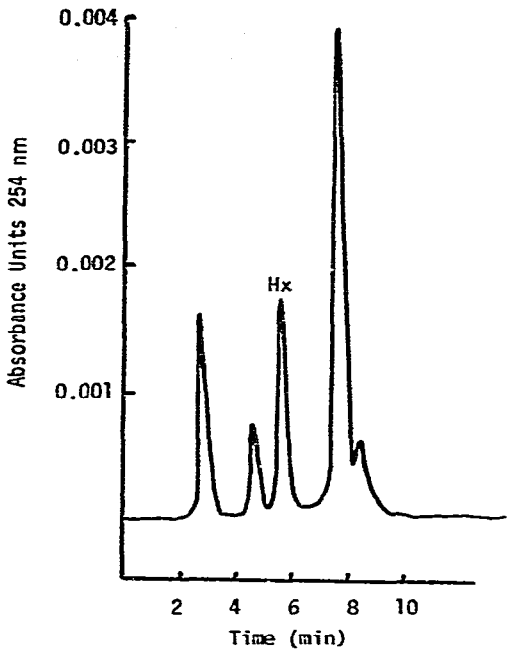

Fig. 2. Separation of hypoxanthine (Hx) from components which co-eluted with hypoxanthine during the initial plasma run. Column, 300 mm × 4 mm I.D. LiChrosorb 10 RP-18; injection volume 100 μl. Running conditions: isocratic elution with 0.025 *M* ammonium acetate pH 5.0 at a flow-rate of 2 ml/min; temperature 23.5°C.

Thymine, oxypurinol and allopurinol analyses

The fractions corresponding to thymine, oxypurinol or allopurinol were subjected to a second chromatographic separation using the same column and isocratic running conditions as for the initial plasma sample (Figs. 3—5). Using an injection volume of 100 μl with run times of 8, 9 and 11 min for thymine, oxypurinol and allopurinol respectively, 40 samples could be assayed within 10 h using the WISP.

Thymidine analysis

Thymidine was assayed using a Zorbax C-8 reversed-phase column running isocratically in 0.05 *M* acetic acid (Fig. 6); a 500-μl injection volume was used. With a run time of 18 min an automated run on the WISP allowed the assay of 40 samples within 16 h.

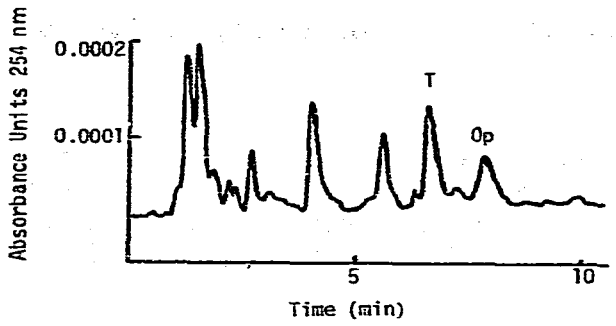

Fig. 3. Separation of thymine from components which co-eluted with thymine during the initial plasma run. Running conditions as for Fig. 1. Peaks: T, thymine and Op, oxypurinol.

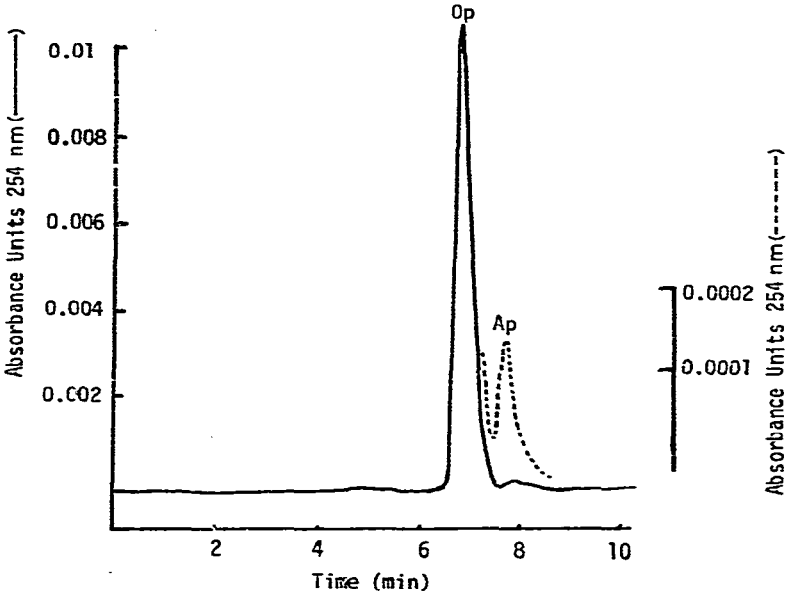

Fig. 4. Separation of oxypurinol from components which co-eluted with oxypurinol during the initial plasma run. Running conditions as for Fig. 1. Peaks: Op, oxypurinol and Ap, allopurinol.

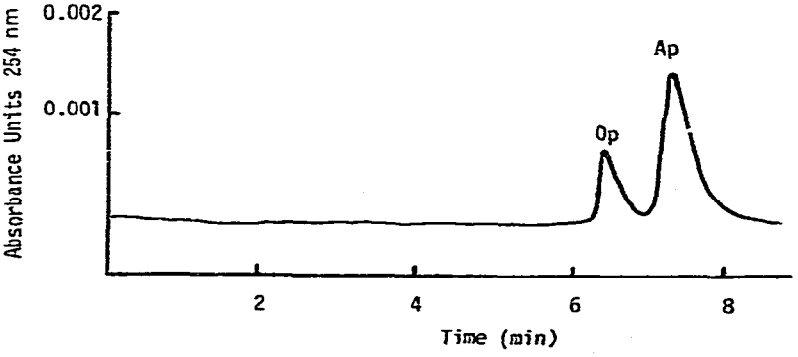

Fig. 5. Separation of allopurinol from components which co-eluted with allopurinol during the initial plasma run. Running conditions as for Fig. 1. Peaks: Op, oxypurinol and Ap, allopurinol.

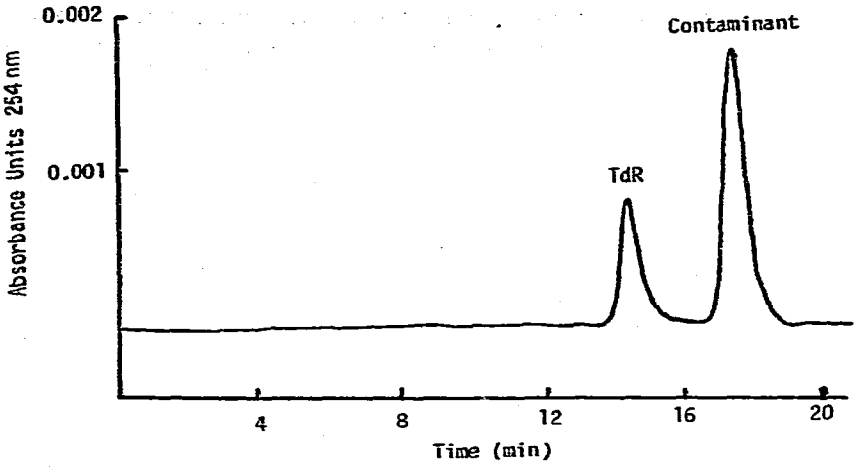

Fig. 6. Separation of thymidine (TdR) from an unidentified component which co-elutes with TdR during the initial plasma run. Column 300 mm × 4 mm I.D. Zorbax C-8; injection volume 500 µl. Running conditions: isocratic elution with 0.05 *M* acetic acid at a flow-rate of 2 ml/min; temperature 23.5°C.

Peak identification and quantification

During the evaluation of the method, fractions were collected from initial runs of plasma samples from mice, patients and healthy human volunteers. The conditions of the second chromatographic separation were varied until we were satisfied that each peak of interest was separated from any contaminant. This involved the comparison of retention times and 254/280 nm absorption ratios with standards under a range of column conditions. When large peaks were obtained, for example in samples from patients with high (ca. 50 µ*M*) circulating hypoxanthine levels it was possible to compare absorption spectra using a Model 450 variable-wavelength detector (Waters Assoc.).

Quantification was achieved by the injection of 0.1—5000-µg samples of standard nucleosides and bases onto appropriate columns running under conditions identical to those used for sample separation.

Equivalent peak areas, defined as the product of the peak area and the full-scale sensitivity, were calculated for each sample and the data subjected to linear regression analysis weighted through the origin (Table I). From these

TABLE I

LINEAR REGRESSION ANALYSIS OF STANDARD CURVES FOR THE QUANTIFICATION OF HYPOXANTHINE, THYMINE, OXYPURINOL, ALLOPURINOL AND THYMIDINE

r = correlation coefficient of line $y = a + bx$; *a* = intercept; *b* = slope; *c* = confidence limits of slope.

	Hypoxanthine	Thymine	Oxypurinol	Allopurinol	Thymidine
r	0.99991	0.991	0.99622	0.9961	0.9988
a	0.09761	−2.5488	−1.27071	−1.44377	0.0368
b	0.07559	0.05325	0.04412	0.04954	0.0309
c	0.00062	0.00285	0.00154	0.00176	0.0007

regression lines calibration factors were obtained allowing the accurate measurement of plasma nucleoside or base levels of 10^{-7} *M* and above.

RESULTS AND DISCUSSION

The separation of a mixture of standards on a μBondapak C-18 reversed-phase column is shown in Fig. 1a. The importance of using patients' samples in evaluating the usefulness of a separation system cannot be over-emphasised, as chromatograms obtained from healthy human plasma samples (Fig. 1b) differ from those obtained from patients' plasma samples (Fig. 1c).

From initial chromatograms of healthy human plasma it is possible to estimate directly the circulating levels of inosine (IR) and thymidine; other components are resolved when appropriate fractions are re-chromatographed. The differences between the patients' and healthy human plasma samples were not uniform: for example approximately half the patients appeared to have an unknown plasma component which co-eluted with thymidine. Fractions collected from patients' initial plasma runs were used to select conditions which would separate thymidine from its unknown contaminants (Fig. 6). This contaminant does not co-elute with a wide range of nucleosides or bases, including the following: adenine, 1-methyladenine, adenosine, 1-methyladenosine, deoxyadenosine, cytosine, 1-methylcytosine, 5-methylcytosine, cytidine, 3-methylcytidine, deoxycytidine, guanine, 7-methylguanine, N-methylguanine, 2-dimethylguanine, guanosine, 1-methylguanosine, 7-methylguanosine, N^2-methylguanosine, deoxyguanosine, thioguanine, 6-mercaptopurine, 6-mercaptopurine riboside, uric acid, xanthine, 1-methylhypoxanthine, 1-methylinosine, 7-methylinosine, uracil, 5-ethyluracil, 5-butyluracil, 5-hexyluracil, 5-fluorouracil, uridine, deoxyuridine, 5-ethyldeoxyuridine, 5-butyldeoxyuridine, 5-hexyldeoxyuridine, pseudouridine.

Circulating levels of inosine were not measured in patients' samples, since they received allopurinol and it is difficult to preclude the presence of allopurinol riboside (ApR, Fig. 1a) as a possible contaminant of the inosine fraction.

Fractions collected on each side of a peak of interest were assayed for any spillover which could occur in samples containing high nucleoside or base levels. This is illustrated by the presence of oxypurinol in the thymine (Fig. 3) and allopurinol (Fig. 5) fractions. As an indication of the sensitivity of the method the thymine and oxypurinol peaks in Fig. 3 and the allopurinol peak in Fig. 5 represent 1.9, 1.43 and 3.66 ng, respectively.

Fig. 7 shows the data obtained from a patient who received an infusion of 27.2 g of thymidine and 2.72 g of inosine over a 47-h period. The plasma from this patient was free of components which co-eluted with thymidine on the initial run, and the close agreement between estimations from the initial run and those obtained from re-running the fractions on the second chromatographic system indicates that this method is both accurate and sensitive (Table II).

The study has revealed features of interest in relation to nucleoside rescue. For example, inspection of Fig. 7 reveals that although high levels of thymidine are infused, only relatively low concentrations (<3 μ*M*) of this nucleoside are detected, while much higher levels of thymine (>10 μ*M*) are achieved during

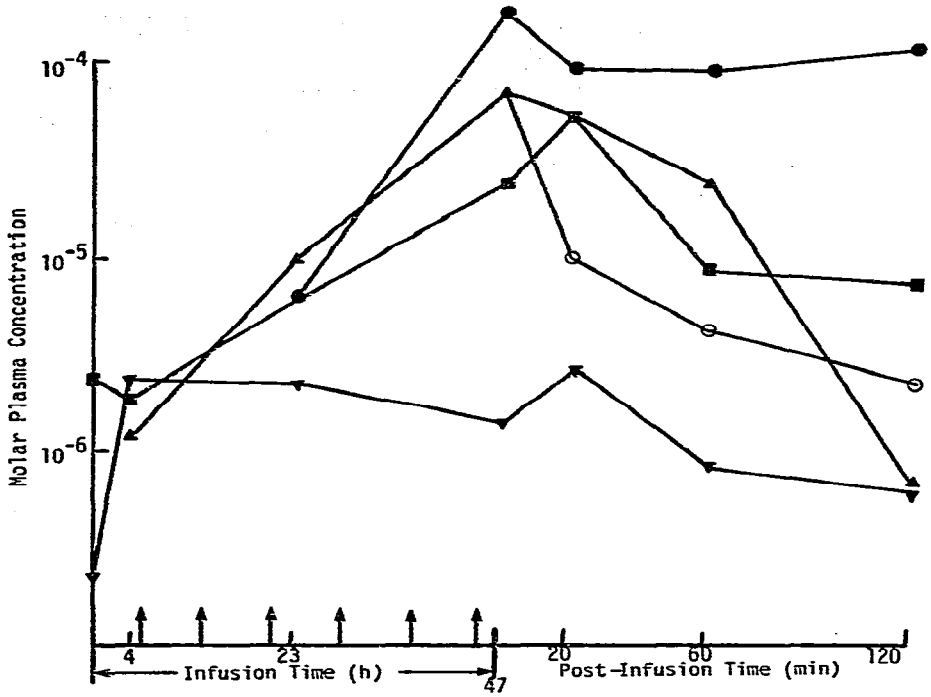

Fig. 7. Plasma concentrations of the nucleosides and bases of interest in a patient receiving an infusion of 2.72 g of inosine and 27.2 g of thymidine over a 47-h period together with 6 doses of allopurinol (200 mg orally at the times indicated by arrows). ■, Hypoxanthine; ▲, thymine; ●, oxypurinol; ○, allopurinol; ▼, thymidine.

TABLE II

COMPARISON OF THYMIDINE LEVELS ESTIMATED FROM INITIAL PLASMA RUNS (A) OR AFTER THE SECOND CHROMATOGRAPHIC SEPARATION (B)

Infusion time (h)	Post-infusion time (min)	Plasma TdR concentration (μM)	
		A	B
0		0.15	–
4		2.31	1.85
23		2.20	2.34
27		—	6.34
47	0	1.45	1.40
	20	2.67	2.50
	60	0.83	0.84
	120	0.60	0.35

the infusion. Clearly, considerable catabolism of thymidine occurs under these conditions, and our results confirm those of other investigators [32,33].

High levels of oxypurinol (10^{-4} *M*) circulate for considerable periods (>6 h) following the oral administration of allopurinol. The consequent inhibition of orotidylate decarboxylase [34] might alter significantly the efficacy of con-

comitantly administered antimetabolites such as 5-fluorouracil [35], azacytidine [36], N(phosphonacetyl)-L-aspartate [37].

Circulating plasma levels of nucleosides and bases were measured in pretreatment plasmas, and in samples obtained from healthy volunteers (Table III). Hypoxanthine levels in the patients' samples fell into two distinct groups, a high group (ca. 50 μM Hx) and a low group (ca. 2.5 μM Hx). Comparison of plasma Hx in the low group and in healthy volunteers (ca. 1.6 μM) shows a significant difference ($p = 0.006$).

TABLE III

CIRCULATING PLASMA LEVELS OF NUCLEOSIDES AND BASES IN HEALTHY HUMAN VOLUNTEERS AND PATIENTS PRIOR TO TREATMENT

	Nucleoside (base)	Number of samples	Mean ± SEM (μM)	Range (μM)
Healthy human plasma	Hx	13	1.2 ± 0.20	0.51—2.81
	T	14	<0.1	<0.1
	IR	14	0.68 ± 0.10	0.26—1.56
	TdR	12	0.43 ± 0.06	0.10—0.92
Patient plasma (pretreatment)	Hx (low)	20	2.62 ± 0.32	0.85—6.50
	Hx (high)	5	52.40 ± 2.30	49.80—64.20
	T	25	<0.1	<0.1
	TdR	12	0.17*	<0.1—2.05

*Median.

In addition to its use in monitoring circulating purines and pyrimidines in patients undergoing clinical study, this method has proved to be highly versatile. Perhaps its most useful minor application has been in the purification of radiolabelled compounds since it allows a simple one-step purification with only limited dilution. In this respect it was particularly useful with relatively unstable isotopes e.g. [^{3}H]thymidine; background counts could be reduced substantially and accurate specific activities determined.

The method is economical with respect to columns, several components being isolated from a single 500-μl injection of biological extract. Indeed the chromatograms shown in Fig. 1 were obtained from a column which had already been used for ten 500-μl plasma samples and was originally prepared using packing material obtained from old columns which had lost their resolution. In addition, as an aqueous isocratic system is used, baseline fluctuations are eliminated, even at very high recorder sensitivities and the fractions collected are of a constant composition lending themselves readily to lyophilisation as the buffers used are volatile.

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