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THE DETERMINATION OF ALLOPURINOL AND OXIPURINOL IN HUMAN PLASMA AND URINE

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SUMMARY

A method is described for allopurinol and oxipurinol assay within human plasma and urine in the range expected during therapy. The method is based on high-performance ion-exchange chromatography following an efficient sample purification step using Chelex-100 resin in the Cu^{2+} form. Linear calibration curves are produced for allopurinol over the range 0.05—10 μmole/l (0.068—1.36 μg/ml) in plasma and 0.005—1 mmole/l (0.68—136 μg/ml) in urine and for oxipurinol 0.5—100 μmole/l (0.076—15.2 μg/ml) in plasma and 0.1—2 mmole/l (15.2—304 μg/ml) in urine.

INTRODUCTION

Formation of uric acid, which is harmful in excess in humans, is much reduced by purine metabolism inhibition by allopurinol (4-hydroxy-3,4-*d*-pyrazolopyrimidine) and its major metabolite oxipurinol (3,4-dihydroxy-3,4-*d*-pyrazolopyrimidine)[1]. Assay procedures other than radioactive dilution [2] suitable for clinical studies of the two compounds had not been published until the preliminary note on an assay procedure based on high-pressure liquid chromatography (HPLC) by Endele and Lettenbauer [3]. A suitable assay, also using HPLC, is described which also discriminates between the naturally occurring purines xanthine and hypoxanthine. The principle is to subject samples, after forced filtration, to a preliminary step of low-pressure ligand-exchange chromatography. HPLC follows but is confined mainly to detection of allopurinol (A), oxipurinol (O), hypoxanthine (H) and xanthine (X). The analytical procedure is equally applicable to urine and plasma.

EXPERIMENTAL

Materials and methods

Ammonia solution (35%, w/v) (Hopkin & Williams, Chadwell Heath, Rom-

ford, Great Britain), ammonium acetate, copper(II) sulphate pentahydrate, glycine, sodium carbonate, sodium hydrogen carbonate and sodium hydroxide solution (2 mole/l) (BDH, Poole, Great Britain) were analytical reagent grade. Brij-35 (polyoxyethylene lauryl ether; BDH) was general purpose reagent grade. Standard pH buffer solutions (Fisons Scientific Apparatus, Loughborough, Great Britain) were used to calibrate the pH meter (Radiometer, Model 26; V.A. Howe, London, Great Britain) in the expanded range. Hypoxanthine and xanthine (Sigma London, Kingston upon Thames, Great Britain) were used as purchased. Allopurinol and oxipurinol (Burroughs Wellcome & Co., Dartford, Great Britain) were obtained in pure form, that is without tablet excipients.

Chelex-100 resin (37—74 μm, 200—400 mesh) (Bio-Rad Labs., Bromley, Great Britain) in the sodium form was first suspended in excess distilled water and acidified with dilute nitric acid to below pH 2.0. The resin was filtered, washed and resuspended in the minimum of 1 mole/l ammonia solution. The resin, now in the ammonium form, was then converted to the Cu^{2+} form by stirring with excess cuproammonium sulphate solution (200 g copper(II) sulphate per litre of 35% (w/v) ammonia solution) for 30 min. The resin was then washed repeatedly with 1 mole/l ammonia solution to remove excess Cu^{2+} ions and then packed in the glass column of the low-pressure ligand-exchange chromatograph as a slurry in this solution.

Aminex A-27 resin in the chloride form (Bio-Rad Labs) was packed in HPLC columns using double-distilled water as the solvent for slurry packing. It was converted to the acetate form by running the chromatographic buffer (1 mole/l ammonium acetate pH 8.70) through the column at normal flow-rate (1 ml/min) for 30 min before use.

Preparation of standards

For HPLC a stock solution of 400 μg/ml of A, O, H and X was prepared. The working HPLC standard solution was made by diluting 25 μl of the stock solution with 25 ml ammonia solution (10 mole/l).

For plasma standards of A and O, stock solutions (34 mg A per 100 ml water and 38 mg O per 100 ml water) were prepared. The calculated volumes of each were added to normal human plasma from volunteers not receiving A or O so that a range of working standards was produced. Standard I contained 20 μmole/l (2.72 μg/ml) A and 100 μmole/l (15.2 μg/ml) O. By serial dilution, standards II, III, IV and V were produced containing the range 10—1.25 μmole/l A and 50—6.25 μmole/l O.

Urine standards were prepared similarly so that the final range was 1—0.05 mmole/l (136—6.8 μg/ml) A and 2.0—0.125 mmole/l (304—19 μg/ml) O.

Apparatus and assay procedure

Plasma and urine filtration. A sample clarification kit (Waters Assoc., Stockport, Great Britain) was assembled omitting the filter but retaining the prefilter; a disposable plastic 2-ml syringe (Brunswick) was substituted for the glass one provided.

One ml plasma diluted to 2 ml with carbonate buffer pH 11.4 containing 1% v/v Brij-35 (30% w/v) was forced through the filter using the syringe. The syringe was disconnected, filled with a further 1 ml of carbonate buffer and again

forced through the filter. Both the initial filtrate and the washings were collected in a 5-ml volumetric flask and their combined volume made up to the mark with the buffer solution (final pH 11.0).

Urine filtration was carried out in the same way as plasma filtration but using 0.5—2.5 ml sample (depending on the expected drug concentration) and omitting Brij-35 from the buffer (final pH 11.0).

Low-pressure ligand-exchange chromatograph. A pressure controller, Superfine Pressure-Stat (Biolabs, Cambridge, Great Britain) introduced nitrogen or compressed air at 1.3—3.5 MN/m^2 (2—5 p.s.i.) into two 2.5-l brown glass bottles each containing a solvent, i.e. 0.01 mole/l carbonate buffer and 10 mole/l ammonia, respectively. The pressurised solvents went via a solvent switching valve (LV4, Pharmacia (GB), Uxbridge, Great Britain) to two more LV4 valves connected as a loop injector system (4-ml volume for plasma or 0.5-ml for urine). The loop injector in turn fed a glass column 65 mm × 6.5 mm I.D. of Chelex-100 Cu^{2+} resin. Interconnecting tubing was 1.5 mm O.D. Buffer flow-rate was 1 ml/min. The column system was triplicated so that three samples could be prepared together.

Injection technique for plasma and urine. The 4-ml sample loop was filled using a 5-ml plastic syringe containing 5 ml filtered plasma. The loop was switched into the carbonate buffer flow. After 15 min the flow was diverted past the loop and 5 min later the solvent valve was switched to the ammonia solution. The next 1 ml eluting from the column was discarded and the following 5 ml which contained the compounds of interest were collected by means of a volumetric flask. Urine was treated in the same way except that a smaller loop was used (usually 500 µl).

The samples purified by ligand-exchange chromatography in the manner described were stored at 2° and generally analysed by HPLC within 24 h by the method described below.

High-pressure liquid chromatography. A Varian 4200 chromatograph (Varian Walton on Thames, Great Britain) was fitted with a fixed-wavelength (254 nm) absorbance detector. The injector was a stop-flow loop injector with 1-ml, 500-µl or 200-µl sample loops. The HPLC pre-column and columns were stainless-steel tubes 6.35 mm (¼ in.) O.D., 4.5 mm I.D., 30 mm and 70 mm long respectively, packed with Aminex A-27 (12—15 µm) anion-exchange resin. The column was totally enclosed in a water jacket and low-dead-volume reducing unions were used at the top and bottom of the columns, and for connecting the pre-column and column with the shortest possible length of 1.5 mm O.D. tubing.

The loop injector was filled with the ligand-exchange treated sample. The size of the loop depended on the concentration of drugs expected but generally 1 ml was used for plasma derived samples and 200 µl for urine derived samples.

The chromatography was then carried out isocratically using 1 mole/l ammonium acetate pH 8.7 at 1 ml/min at 71° as eluting solvent and an inlet pressure of 10—11.3 MN/m^2 (1500—1700 p.s.i.). Peak areas were integrated and recorded by a HP 3352 computer (Hewlett-Packard, Winnersh, Great Britain).

Calibration method. The HPLC chromatograph performance was always checked by running the HPLC H, A, X, O stock solution before any samples. The three Chelex columns were calibrated by running the range of prepared standards through the described procedure. Unknown samples were analysed in the same way as the prepared standards.

RESULTS

Fig. 1a shows a typical chromatogram of the stock H (R_t = 4.45 min), X (9.10 min), A (5.98 min) and O (17.08 min) solution. The elution order was always reproducible with good resolution, and readily interpreted by the integrator system. No major peaks were seen after the elution of O. The HPLC column (including pre-column) usually gave a height equivalent to theoretical plate (HETP) value of 0.125—0.0625 mm. When distortion of the X and O peaks oc-

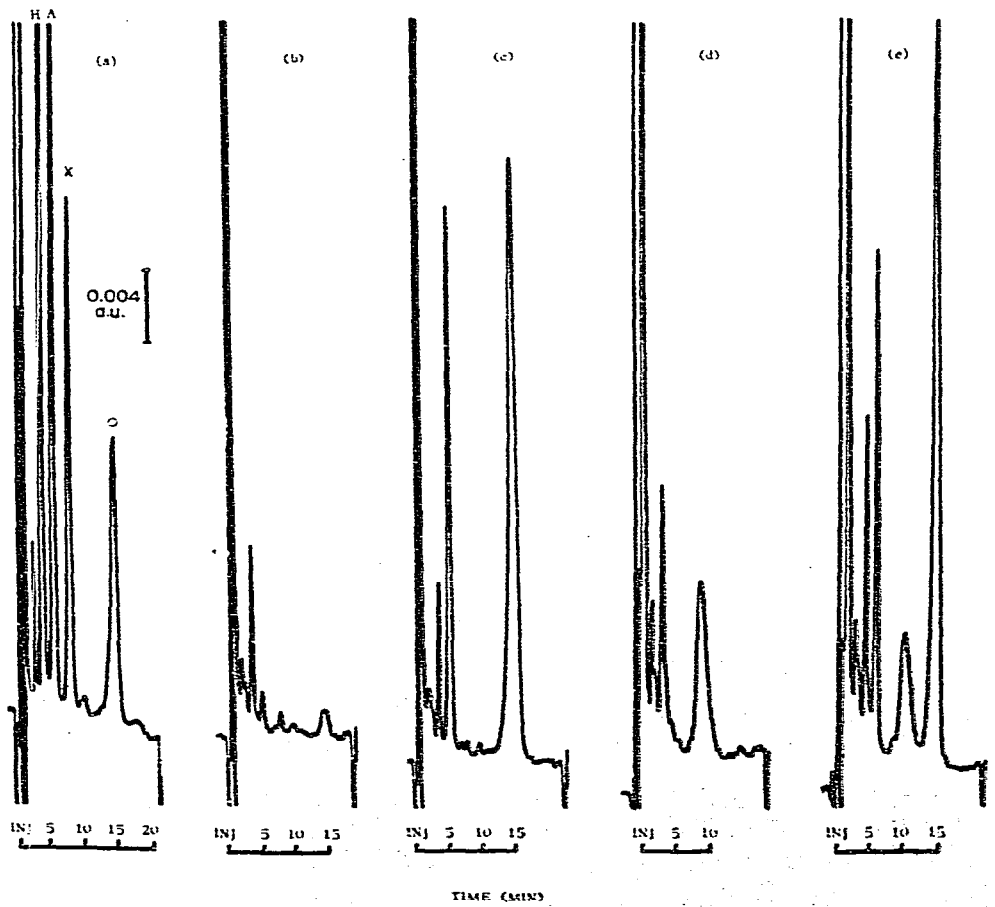

Fig. 1. Typical chromatograms of (a) a standard containing hypoxanthine (H), xanthine (X), allopurinol (A) and oxipurinol (O), (b) blank urine, (c) standard urine, (d) blank plasma and (e) standard plasma.

curred, changing the pre-column restored column performance.

Figs. 1b, c, d and e are typical chromatograms of blank urine, standard urine, blank plasma and standard plasma, respectively, showing an absence of interfering peaks.

When the plasma and urine standards were analysed, the data were collected and used to prepare calibration curves which were subjected to least squares regression analysis and tested for linearity. When the intercepts of the calibration lines did not significantly differ from zero, the least squares line, forced through the origin, was used. Urine calibration lines were all linear and no intercept differed significantly from zero ($2P>0.05$). Table I summarises the statistical results for urine. The three slopes for A on Chelex columns 1, 2 and 3 did not differ significantly from each other and their common slope was highly significant ($2P<0.001$). Fig. 2 shows the common line together with its 95% confidence limits. The three slopes for O differed significantly but consistently from each other and this was taken into account in the quantitation of O in urine.

TABLE I

CALIBRATION LINES FROM URINE FOR ALLOPURINOL AND OXIPURINOL, FORCED THROUGH THE ORIGIN, FROM CHELEX COLUMNS 1, 2 AND 3

	Chelex column	Calibration line*	R^2
Allopurinol	1	$y = 60.839x$	0.990
	2	$y = 64.007x$	0.989
	3	$y = 61.862x$	0.995
Common regression line		$y = 62.329x$	0.991
Oxipurinol	1	$y = 40.507x$	0.974
	2	$y = 45.223x$	0.976
	3	$y = 41.869x$	0.993

No common regression derived as slopes are significantly different.

*y = Integrated peak area (mV·sec); x = concentration (mmole/l).

Plasma calibration lines were all linear and no intercept differed significantly from zero. Three slopes each for A and O were all linear and no intercept differed significantly from zero and the common slope was highly significant ($2P<0.001$). Table II summarises the statistical results for plasma.

Inclusion of the standard of the highest concentration reduced the slope of the A calibration lines, which did not then always pass through the origin. Hence the method was only valid for concentrations up to 10 μmole/l where the calibration was linear. Samples with original concentrations higher than this must first be diluted.

A 0—12-h urine sample from a healthy female volunteer who had taken 300 mg A (as a standard tablet) was repeatedly analysed. For each analysis 0.5 ml of urine was filtered, 5 ml were collected from the Chelex column and 500 μl of this eluate were injected on to the HPLC column. The results show a mean

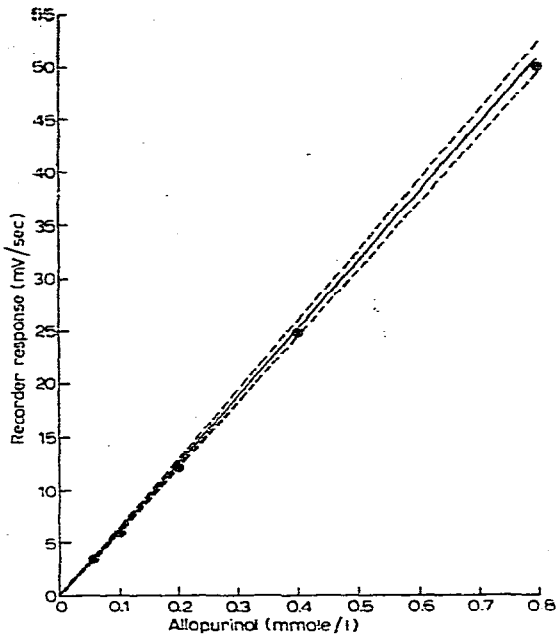

Fig. 2. A typical calibration line showing the common slope (with 95% confidence limits) from Chelex columns 1, 2 and 3 for allopurinol in repeatedly analysed (n = 10) urine standards.

TABLE II

CALIBRATION LINES FROM PLASMA FOR ALLOPURINOL AND OXIPURINOL, FORCED THROUGH THE ORIGIN FROM CHELEX COLUMNS 1, 2 AND 3

	Chelex column	Calibration line*	R^2	
Allopurinol	1	y = 2.012x	0.998	n.s. diff.**
	2	y = 2.043x	0.996	
	3	y = 1.960x	0.992	
Common regression line		y = 2.010x	0.996	
Oxipurinol	1	y = 1.580x	0.995	n.s. diff.
	2	y = 1.528x	0.996	
	3	y = 1.574x	0.997	
Common regression line		y = 1.559x	0.996	

*y = integrated peak area (mV·sec); x = concentration (mmole/l).
**ns diff. = not significantly different.

value of 0.3133 mmole/l (S.D. 0.01451) for A and 0.6552 mmole/l (S.D. 0.0226) for O. Fig. 3 is a typical plasma profile from a patient who had received 300 mg A showing the rapid absorption and elimination of A, the formation and slow elimination of O and the concentration of each drug usually encountered after a single oral dose in man.

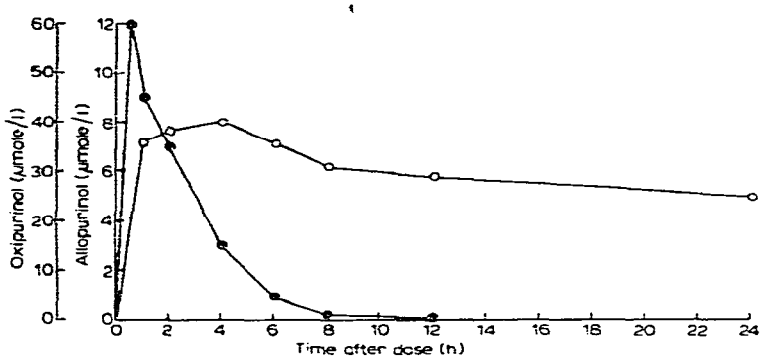

Fig. 3. Typical plasma profile of allopurinol (●) and oxipurinol (○) from a patient who received one 300-mg dose of allopurinol orally.

DISCUSSION

Several methods of determining A and O have been published but each has limitations as a routine analytical technique in biological fluids. Methods based on electrophoresis and polarography lack the requisite sensitivity and selectivity. Anion-exchange chromatography of purine nucleotides is well documented [4] and was exploited successfully with HPLC [3, 6]. However, when applied to the purine bases H, A, X and O there was the complication caused by the many other anions normally present in biological fluids. The peaks of interest elute soon after the void volume so that an unnecessary delay of about 6 h is encountered while waiting for the elution of the later peaks (nucleotides, etc.). Even the use of complex gradients with expensive high-performance ion-exchange packings gave an analysis time of 40 min but reproducibility and column life suffered. Attempts to simplify these procedures proved unsuccessful as no way of selectively removing the later eluting peaks was found. Attempts to concentrate the sample after removing protein (e.g., ultrafiltration, trichloroacetic acid, etc.) led to variable recovery. An internal standard as recently described by Endele and Lettenbauer [3] overcame this variability to some extent but the basic problem of the numerous other anions persists with the associated problems such as short column life.

These shortcomings prompted the search for selective purification for A and O by adsorption. A xanthine oxidase competitive binding technique was rejected because labelled material would be needed routinely and A would be a substrate for the production of O. The method now recommended originated from the observation that purines form an almost insoluble Cu^{2+} complex. As described above, when diluted raw plasma or urine passes down a column of Cu^{2+} Chelex-100 in an analogous manner to that described by Siegel and Degens [6], followed by a stepwise elution with ammonia, H, A, X and O are exclusively retained in the 10 mole/l ammonia fraction. On directly applying this fraction to the ion-exchange column no major peaks eluted after O showing that neither uric acid [7] nor the other late peaks were retained with H, A, X or O by the Cu^{2+} Chelex. The HPLC ion-exchange system was so simplified by the inclusion of ligand exchange that a total analysis time of 25—30 min was possible in the

isocratic mode as opposed to a minimum of 40 min with a gradient elution system. Likewise the ligand-exchange system was straightforward and the overall throughput using the two-column system was better than that of either system alone.

The use of copper Chelex-100 is not itself without problems; other authors [6, 8, 9] have complained of Cu^{2+} ion bleed from the columns especially after the running of biological samples. However the extent of the Cu^{2+} ion bleed diminishes with use and can be attenuated to an acceptable level if the column is initially treated with 0.5 ml injection of 10 μg/ml glycine solution. The recoveries of H, X, A and O were only quantitative on glycine-treated or "old" columns and the recoveries of X and O were only quantitative at pH 11.0.

Some system overloading can be detected (>10 μmole/l A) but in practice these higher levels can be measured by simply using less sample volume in the filtration step. When the lower sample level of 0.5 ml was used for urine, Chelex column life was longer and gave fewer problems (e.g. blocked frits) than the higher level.

The binding characteristics of H and X to Chelex were observed to be similar to A and O; this suggests that the method can be adapted to measure these purines. Indeed, where H and X are to be measured in biological fluids not containing A or O, these latter compounds may be used as internal standards. However, as H and X are always present in biological fluids, "blank" samples would be difficult to obtain and such a method would only detect changes in level.

In conclusion, the method is suitable for analysing A in the range 0.05—10 μmole/l (0.0068—1.36 μg/ml) in plasma and 0.005—1 mmole/l (0.68—136 μg/ml) in urine and O in the range 0.5—100 μmole/l (0.076—15.2 μg/ml) in plasma and 0.1—2 mmole/l (15.2—304 μg/ml) in urine.

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