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## Note

# Simultaneous determination of allopurinol, oxipurinol, hypoxanthine and xanthine in biological fluids by high-performance liquid chromatography

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Allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine] is increasingly used for the treatment of hyperuricaemia. Allopurinol and its major metabolite, oxipurinol, are potent inhibitors of xanthine oxidase, the enzyme that converts hypoxanthine to xanthine, and xanthine to uric acid. As a result, allopurinol decreases uric acid production and increases hypoxanthine and xanthine formation in the blood and urine of patients treated with this drug. Several high-performance liquid chromatographic (HPLC) methods for the determination of allopurinol and oxipurinol have been published [1—5]. In this paper is presented an HPLC procedure for the simultaneous determination of allopurinol, oxipurinol, hypoxanthine and xanthine, and its application to the analysis of plasma and urine from hyperuricaemic patients treated with allopurinol.

## EXPERIMENTAL

### *Reagents*

Allopurinol, oxipurinol, hypoxanthine and xanthine were purchased from Sigma (St Louis, MO, U.S.A.). 9-Methylxanthine was obtained from Fluka

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(Buchs, Switzerland). Potassium dihydrogen phosphate, orthophosphoric acid, trichloroacetic acid and perchloric acid were obtained from Merck (Darmstadt, F.R.G.).

*Apparatus and chromatographic conditions*

Chromatographic analyses were done with an integrated unit, an SP 8000 high-performance liquid chromatograph (Spectra Physics, Orsay les Ullis, France). The column (15 cm × 4.6 mm I.D.) and the precolumn (5 cm × 4.6 mm I.D.), used as a guard column, were packed with Hypersil ODS, 3 μm (Shandon, Cheshire, U.K.) by the slurry packing technique as described by Coq et al. [6]. The mobile phase consisted of 0.02 *M* potassium dihydrogen phosphate, the pH of which was adjusted to 3.65 with orthophosphoric acid. The flow-rate was 1.5 ml/min. Detection was carried out at 254 nm.

*Sample collection and treatment*

One millilitre of blood was collected in a heparinized tube. In accordance with our previous findings, this tube was immediately centrifuged to prevent an increase of hypoxanthine concentration in plasma samples left in contact with the erythrocytes [7]. Plasma was decanted and stored at −20° C until analysis. A 500-μl aliquot of plasma was spiked with the internal standard (9-methylxanthine, 12.5 μmol/l). The samples were then deproteinised by 150 μl of trichloroacetic acid (12%) or 50 μl of perchloric acid (35%).

Twenty-four-hour urine samples were collected and stored at −20° C. Urine samples were analysed without further treatment.

## RESULTS AND DISCUSSION

A chromatogram obtained from a standard solution containing hypoxanthine, xanthine, 9-methylxanthine, oxipurinol and allopurinol is shown in Fig. 1. Fig. 2a and c show typical chromatograms of blank plasma and blank urine, respectively.

Chromatograms of plasma and urine samples from a patient under allopurinol therapy, 300 mg once daily, are presented in Fig. 2b and d, respectively. As can be seen by comparison of chromatograms from blank samples (Fig. 2a and c) and from biological samples (Fig. 2b and d) there are no endogenous interfering peaks at retention times corresponding to those of allopurinol and oxipurinol. Furthermore, the chromatograms of biological samples show that uric acid is separated from compounds of interest in this study.

Calibration curves were linear from 0.15 to 20 mg/l for allopurinol and oxipurinol and from 0.50 to 50 μmol/l for hypoxanthine and xanthine with intercepts not significantly different from zero. Furthermore, slopes corresponding to aqueous solutions and plasma are identical; this confirms that there was no loss of any of the compounds under study during deproteinisation.

Under the conditions employed in this study, the minimum quantity detectable is 1.5 ng for allopurinol and oxipurinol, 2.5 pmol for hypoxanthine and 5.0 pmol for xanthine.

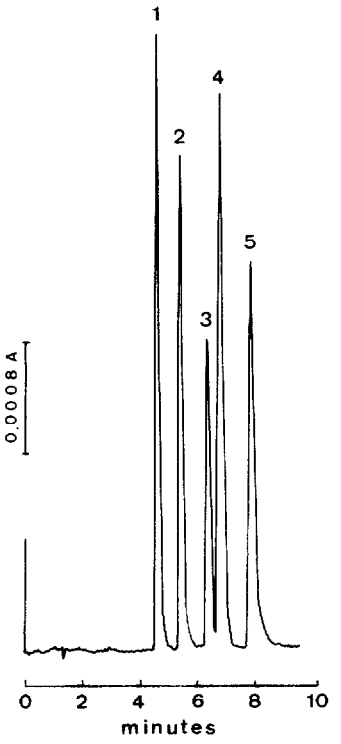


Fig. 1. Chromatogram of a standard solution containing (1) hypoxanthine (10.0 μmol/l), (2) xanthine (10.0 μmol/l), (3) 9-methylxanthine (12.5 μmol/l), (4) oxipurinol (4.0 mg/l) and (5) allopurinol (2.5 mg/l). Injection volume: 10 μl. Column: Hypersil ODS, 3 μm. Mobile phase: 0.02 *M* potassium dihydrogen phosphate, pH 3.65. Flow-rate: 1.5 ml/min. Detection: 254 nm.

TABLE I

REPRODUCIBILITY AND ACCURACY OF ALLOPURINOL AND OXIPURINOL DETERMINATION IN SPIKED INTRA-ASSAY PLASMA

| | Amount added (mg/l) | Amount found (mg/l; mean ± S.D.; $n$ = 5) | C.V. (%) |
|---|---|---|---|
| Allopurinol | 0.5 | 0.48 ± 0.02 | 4.2 |
| | 2.0 | 2.06 ± 0.05 | 2.4 |
| | 5.0 | 5.09 ± 0.11 | 2.2 |
| Oxipurinol | 0.5 | 0.49 ± 0.02 | 4.1 |
| | 2.0 | 2.01 ± 0.08 | 3.9 |
| | 5.0 | 5.01 ± 0.15 | 2.9 |

Reproducibility and accuracy were determined on spiked plasma. The intra-assay results for allopurinol and oxipurinol are given in Table I. The inter-assay was performed by analysing spiked plasma samples on different days over one week ($n$ = 5). The inter-assay coefficient of variation for the analysis of allopurinol and oxipurinol over the concentration range 0.5—5 mg/l was

found to be about 3% for both allopurinol and oxipurinol. The results obtained for hypoxanthine and xanthine have been listed in a paper published previously [8]; for these two compounds, the coefficient of variation was found to be about 1.5%.

Contrary to some methods published [4, 5] for the determination of allopurinol and oxipurinol, a rapid sample clean-up procedure (plasma deproteinisation) was used in order to obtain baseline separations, symmetric peak shape and constant retention times.

This paper describes a rapid and sensitive HPLC method for the monitoring of allopurinol and oxipurinol concentrations in plasma and urine to obtain a

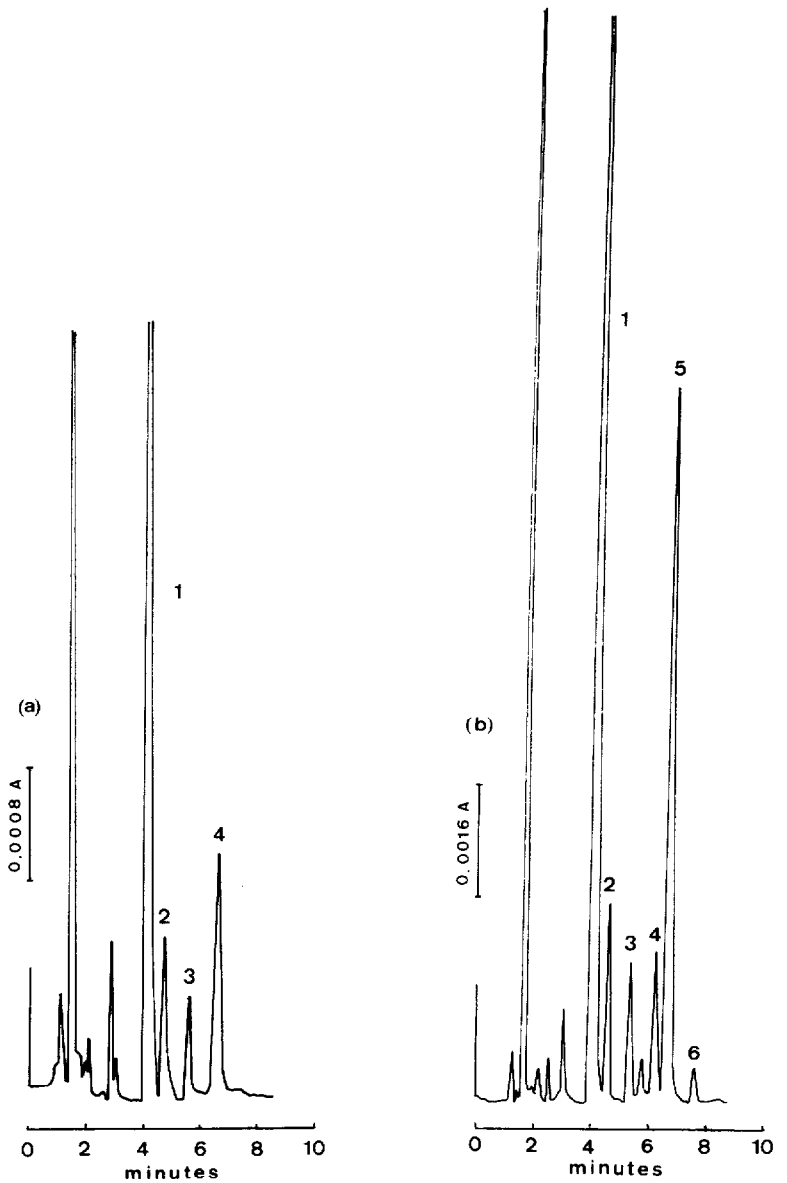


Fig. 2.

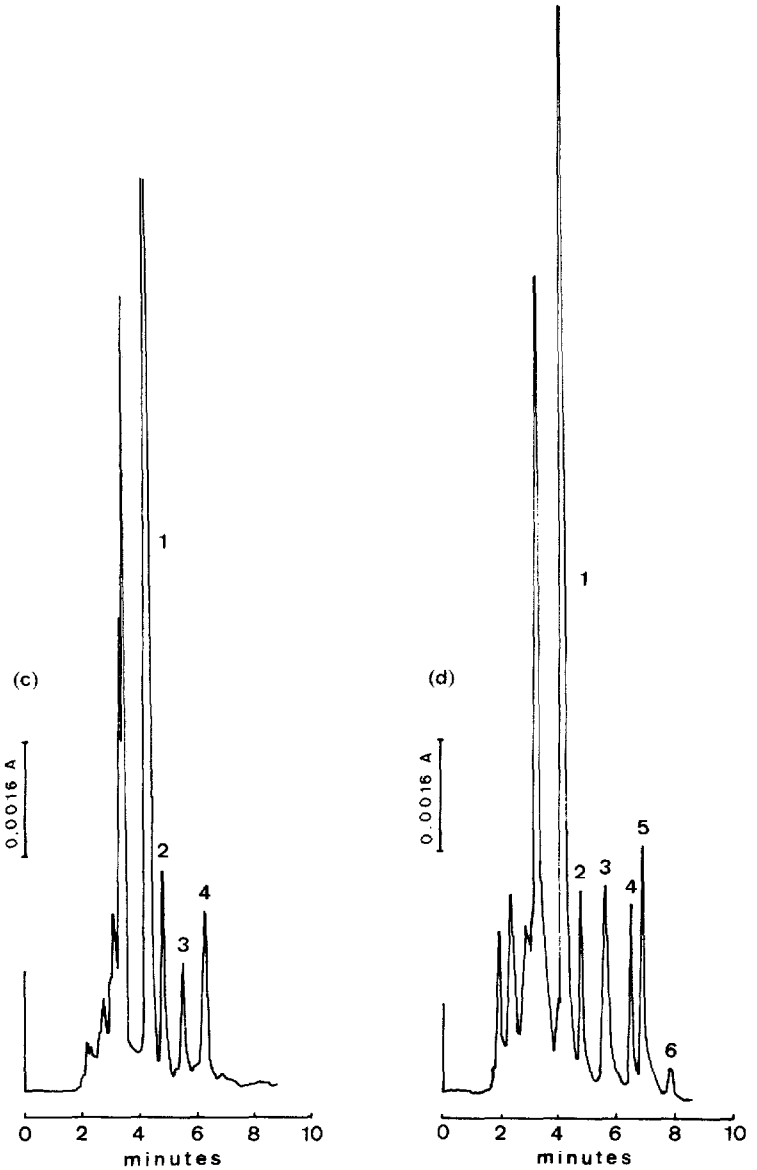


Fig. 2. Chromatograms of (a) blank plasma, (b) plasma sample from a patient under allopurinol therapy, 300 mg once daily, (c) blank urine diluted five times, and (d) urine sample, diluted ten times, from the same patient under allopurinol therapy. Peaks: 1 = uric acid; 2 = hypoxanthine [(a) 1.5 μmol/l, (b) 3.5 μmol/l, (c) 29.5 μmol/l (44 μmol per 24 h), (d) 52.0 μmol/l (99 μmol per 24 h)]; 3 = xanthine [(a) 2.0 μmol/l, (b) 5.5 μmol/l, (c) 25.0 μmol/l (37.5 μmol per 24 h), (d) 90.0 μmol/l (171 μmol per 24 h)]; 4 = 9-methyl xanthine (12.5 μmol/l); 5 = oxipurinol [(b) 12.0 mg/l, (d) 35.0 mg/l (66.5 mg per 24 h)]; 6 = allopurinol [(b) 0.5 mg/l, (d) 4.0 mg/l (7.6 mg per 24 h)]. The concentrations of the compounds of interest in urine samples are given taking dilution into account.

significant pharmacological effect and also to prevent toxic side-effects of the drug, in particular, the formation of oxipurinol stones in patients with renal failure.

Using this method, we observed that the hypoxanthine and xanthine levels in plasma and especially in urine from patients under allopurinol therapy are higher than those obtained in our study of healthy subjects [7]. With this HPLC method, the effect of allopurinol treatment on hypoxanthine and xanthine levels in biological fluids can easily be studied, while monitoring allopurinol levels in patients under allopurinol therapy.

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