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Note

Determination of allopurinol and oxipurinol in biological fluids by high-performance liquid chromatography*

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Allopurinol (4-hydroxy-3,4-*d*-pyrazolopyrimidine), a structural analogue of hypoxanthine, is a useful drug for the treatment of gout and hyperuricemia [1—3]. Allopurinol and its major metabolite oxipurinol (3,4-dihydroxy-3,4-*d*-pyrazolopyrimidine) decrease uric acid production by inhibiting xanthine oxidase, the enzyme that converts hypoxanthine to xanthine and xanthine to uric acid [4, 5]. Additionally, allopurinol and oxipurinol depress de novo purine biosynthesis through feedback inhibition of amidophosphoribosyltransferase and by depletion of the essential substrate, phosphoribosylpyrophosphate [6, 7].

Adverse effects of allopurinol therapy are reported to occur more frequently in patients with renal insufficiency [8]. Therefore, a knowledge of blood levels of allopurinol and oxipurinol will be helpful in establishing adequate drug dosage, especially in patients with renal impairment.

Previously described methods for the determination of allopurinol and oxipurinol include radioactive detection after paper or column chromatographic separation [9], column chromatography on Dowex resins [9, 10], gas chromatography [11], mass spectrometry [12], and direct electrochemical determination [13]. A competitive binding assay [14], photometric enzyme-inhibition assays [15, 16], and, more recently, a fluorimetric enzyme-inhibition test [17] have been described. Several high-performance liquid chromatographic (HPLC) methods for the determination of allopurinol and oxipurinol in biological fluids have been published [18—26]. However, their applicability to clinical monitoring remains questionable. The disadvantages of these methods

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include difficult sample preparation [19, 25], long analysis time [18, 19, 26], insufficient sensitivity [23, 24] or selectivity [25], and restricted applicability for allopurinol [20, 22] or serum [21, 26] only.

Therefore, a rapid, simple and sensitive HPLC assay procedure for allopurinol and its active metabolite, oxipurinol, in plasma and urine, which is suitable for clinical studies, has been developed in our laboratory.

EXPERIMENTAL

Chemicals

Allopurinol, oxipurinol and uric acid were purchased from Henning, Berlin, G.F.R. 6-Thioguanine, guanine and 8-azaguanine were delivered from Burroughs Wellcome, London, Great Britain. All other substances were analytical grade reagents and were used without further purification.

Apparatus

The high-performance liquid chromatograph was equipped with a Gynkotek HPLC pump Model 600/200 (Gynkotek, Munich, G.F.R.), a modified automatic sample injector ASI 45 (Kontron, Eching, G.F.R.), a variable-wavelength ultraviolet detector Uvikon 720 LC (Kontron, Eching, G.F.R.), a computing integrator SP 4100 (Spectra-Physics, Darmstadt, G.F.R.), and a two-channel electronic recorder BD9 (Kipp and Zonen, Kronberg/Ts., G.F.R.).

Assay procedure

A short-alkyl reversed-phase material (SAS-Hypersil, 5 μm, 30 cm × 4.1 mm I.D.) was used as stationary phase. The eluent was prepared by mixing 190 ml of 0.1 *M* citric acid monohydrate with 810 ml of 0.2 *M* disodium phosphate and 2 l of distilled water. The flow-rate was 2.0 ml/min at a back pressure of 270 bar and at room temperature. Detection was set at 0.02 a.u.f.s. and at 252 nm, the absorption maximum of allopurinol at pH 7 [27].

Blood samples (1 ml) were taken into heparinized tubes and centrifuged at 8000 *g* for 5 min. Plasma was withdrawn, diluted 1:2 in the eluent, and injected into the chromatograph (20-μl samples). Plasma not immediately analyzed was stored frozen at −16°C. Urine samples were diluted 1:20 in distilled water before aliquots of 20 μl were chromatographed.

For quantitation the areas under the curves were computed by an integrator. Calibration was performed by the method of external standardization. Each sample was analyzed in duplicate. A third analysis was performed if the peak areas of the compounds differed by more than 5%.

Protein binding

For determination of protein binding, ultrafiltration was performed using a Model MM 302 ultrafiltration system (Amicon, Düren, G.F.R.) and Type PM 10 Diaflo® membranes. In controls there was no adsorption of allopurinol and oxipurinol to this membrane.

Stability

Stability of allopurinol and oxipurinol in human blood was tested in heparinized fresh blood samples, which were spiked with both compounds (1 μg/ml). Each sample was gently shaken at 37°C. After various time intervals aliquots were withdrawn, centrifuged and plasma samples subjected to HPLC.

RESULTS AND DISCUSSION

SAS-Hypersil was found to be a suitable reversed-phase material with regard to selectivity and stability. Since the quality of the SAS-Hypersil column did not noticeably decline with untreated plasma or urine (up to 200 injections of 20-μl samples), samples of plasma or urine were analyzed directly avoiding all deproteinization and extraction procedures. Optimal resolution was obtained with a citrate—phosphate buffer eluent (50 m*M*, pH 7.0). The addition of the citrate component provided sharp, symmetrical and well-defined peaks of allopurinol and oxipurinol (Fig. 1). Constituents of plasma (Fig. 1d and e) or urine (Fig. 1f and g) did not interfere with the resolution of either compounds.

The retention times of allopurinol (10 min) and oxipurinol (7.8 min) were quite stable with a relative standard deviation of less than 5%, as demonstrated from day to day with standard test solutions.

Separation was not disturbed by other purine analogues, such as uric acid,

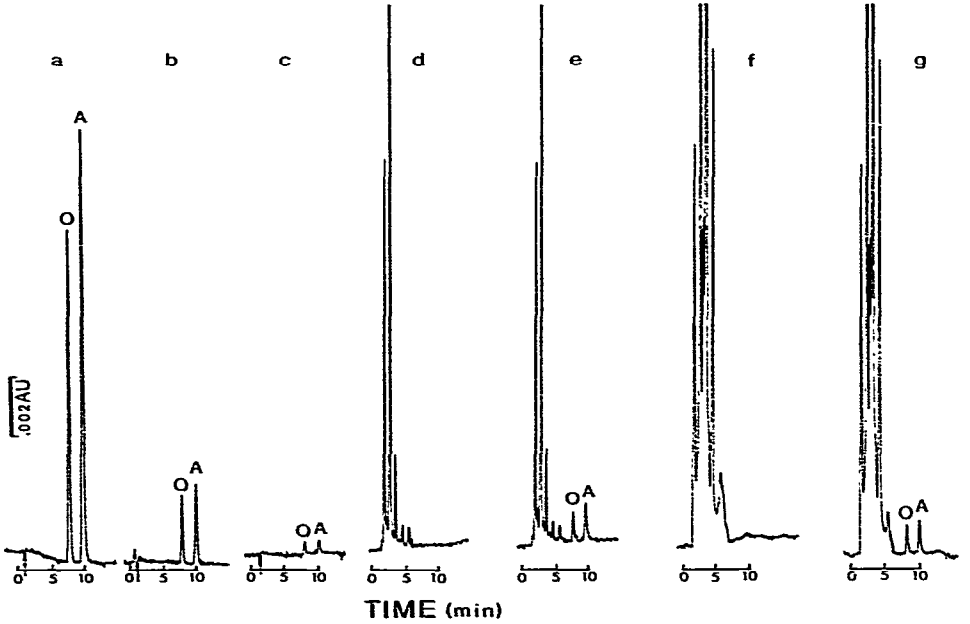

Fig. 1. Chromatograms of standards containing (a) 2.5, (b) 0.5, (c) 0.1 μg of allopurinol (A) and oxipurinol (O) per ml, (d) blank plasma, (e) standard plasma, (f) blank urine, and (g) standard urine. Plasma was diluted 1:2 and urine 1:20 in distilled water. Stationary phase: SAS-Hypersil (5 μm, 30 × 0.41 cm I.D.). Mobile phase: citrate—phosphate buffer (50 m*M*, pH 7.0). Sample volume: 20 μl. Flow-rate: 2 ml/min. Back pressure: 270 bar. Room temperature. Detection at 252 nm.

xanthine, hypoxanthine, guanine, 8-azaguanine, 6-thioguanine, and 6-mercaptopurine. No interfering peaks were found in the plasma of patients 2 h after application of the following drugs: acetylsalicylic acid, azathioprine, benzbromarone, caffeine, cotrimoxazole, cytarabine, diazepam, dihydralazine, dipyridamole, fluorouracil, methotrexate, procarbazine, propranolol, spironolactone, sulfinpyrazone, aminophylline, 6-thioguanine, and 6-mercaptopurine.

Calibration curves for allopurinol and oxipurinol were linear from 0.1 to 50 μg/ml with intercepts not significantly different from zero. The limit of detection was 0.1 μg/ml with a coefficient of variation of less than 5% for both compounds. The day-to-day precision as determined on six consecutive days for frozen plasma samples (1 μg/ml) was found to be 6.9% for allopurinol and 7.8% for oxipurinol.

Recovery from spiked samples of plasma or urine was 97—102% for both compounds when compared with the peak areas of aqueous standards. Since recovery from plasma or urine was highly reproducible and preliminary-clean-up procedures were eliminated, the method of external standardization was used for quantitation. Stability of allopurinol and oxipurinol in human blood, plasma and urine at 37°C was excellent. Contrary to the preliminary report of Kramer and Feldman [28], no evidence of erythrocyte metabolism of the drug was observed during the incubation period (2 h).

The plasma protein binding of allopurinol was found to be 2.0 ± 3.7% (mean ± S.D.) for the concentration range 0.5—50 μg/ml. In contrast to Elion et al. [9], who reported no significant protein binding of either allopurinol or oxi-

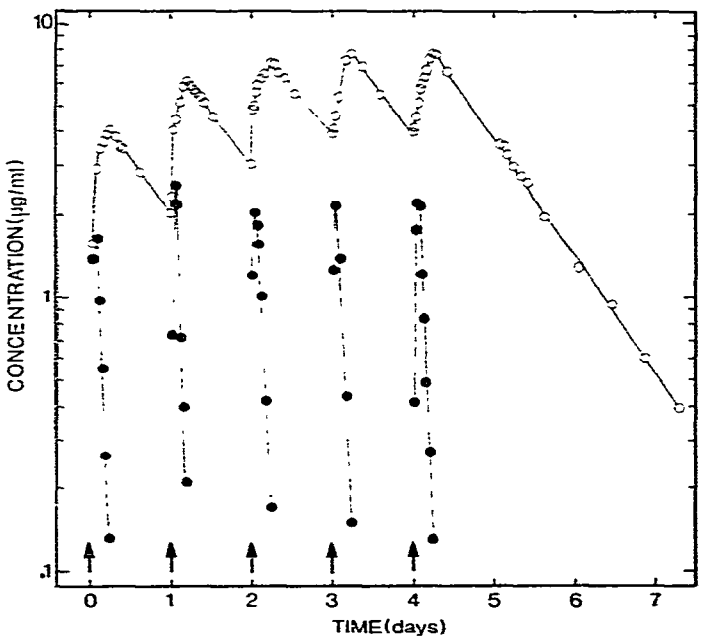

Fig. 2. Time course of plasma concentration of allopurinol (•) and oxipurinol (○) during and after oral application (↑) of 300 mg of allopurinol (3.2 mg/kg) on five consecutive days. Each point represents the mean value of two determinations. A third analysis was performed if the difference was more than 5%.

purinol, the binding of oxipurinol to plasma proteins was 16.8 ± 4.4% (mean ± S.D.).

The HPLC method was applied to pharmacokinetic studies in plasma (Fig. 2) and urine (Fig. 3). The plasma kinetics after oral application of 300 mg of allopurinol on five consecutive days demonstrate the rapid disappearance of allopurinol from plasma, with a half-time of 1.13 ± 0.13 h (mean ± S.E., $n = 5$ days). The plasma levels (1.5—8 μg/ml) and half-time of oxipurinol (18.45 ± 1.32 h) were comparable to those found by others [9, 16, 20]. The urinary recovery of intact allopurinol was 23% and that of oxipurinol 71% of the total dose administered.

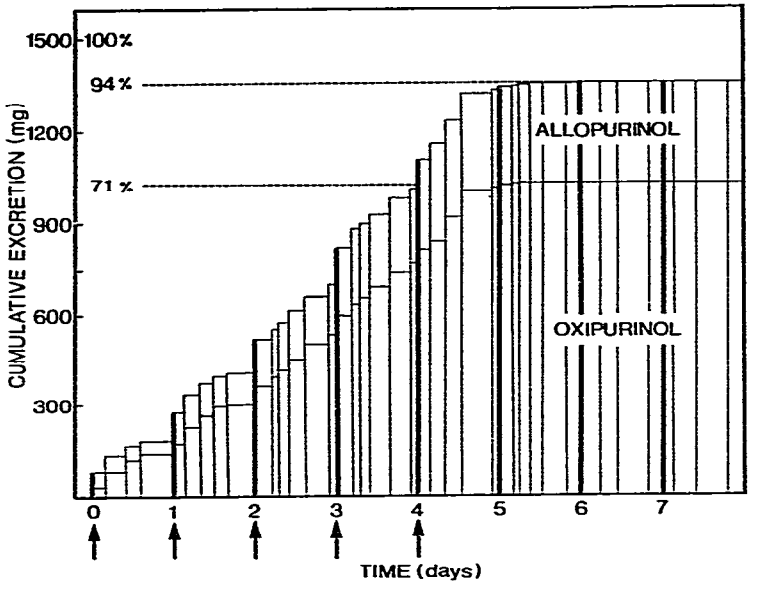

Fig. 3. Cumulative excretion of allopurinol and oxipurinol in urine. A total dose of 1500 mg of allopurinol was orally administered, given in five single doses of 300 mg on five consecutive days (↑).

In summary, a rapid and simple HPLC assay for the analysis of the hypouricemics allopurinol and oxipurinol in plasma and urine is described. Without time-consuming clean-up procedures, plasma and urine samples were analyzed directly by isocratic reversed-phase chromatography providing complete separation from constituents of plasma or urine. Both compounds can be precisely determined in human plasma and urine in concentration ranges usually encountered after therapy with allopurinol. This methods offers significant advantages in terms of rapidity, simplicity, specificity and reproducibility over previously published methods [9—26].

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