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Simultaneous determination of allopurinol and oxypurinol by liquid chromatography using immobilized xanthine oxidase with electrochemical detection

EUGENE J. EISENBERG*, PHIL CONZENTINO, GARY G. LIVERSIDGE and KENNETH C. CUNDY

Department of Drug Delivery, Sterling Research Group, Great Valley, PA 19355 (U.S.A.)

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ABSTRACT

A novel liquid chromatographic method using an immobilized xanthine oxidase reactor and an electrochemical detector was developed for the simultaneous determination of allopurinol and oxypurinol in rat plasma, intestinal wash and bile. Xanthine oxidase was immobilized on 5-μm aldehyde silica (prepacked into a 2 mm × 10 mm cartridge) in a simple procedure. Allopurinol eluted from an analytical column was converted to oxypurinol in the enzyme reactor with the eluent as the reaction medium and detected with high selectivity using an amperometric detector with a glassy carbon electrode at the applied potential of +0.85 V. High specificity of the enzymatic reaction combined with selectivity of the electrochemical detection eliminated the need for an extensive sample preparation. The assay was linear in the range 15–500 ng/ml of rat plasma, intestinal wash and bile with a low limit of detection of 10 pg on-column (signal-to-noise ratio = 4) for both allopurinol and oxypurinol.

INTRODUCTION

Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine), a structural analogue of hypoxanthine, is a potent xanthine oxidase inhibitor and is widely used for the treatment of gout and hyperuricemia [1]. Since the differential absorption of allopurinol from various sites of the gastrointestinal tract has been reported [2], it was selected as a model compound for an improved *in situ* intestinal absorption rat model developed in our laboratory [3].

In this modified *in situ* model, the rate of disappearance of the drug from intestinal lumen is monitored simultaneously with its systemic, pre- and post-hepatic plasma levels as well as its bile concentrations. Allopurinol is rapidly metabolized in rat tissue and blood. Its major metabolite, oxypurinol (3,4-di-hydroxypyrazolo [3,4-*d*]pyrimidine), is a product of enzymatic oxidation and is also an inhibitor of xanthine oxidase [4]. Since the half-life of oxypurinol is considerably longer than that of allopurinol, it is used as an indicator of absorption of the parent drug. A simple and accurate method is required for the simultaneous determination of both allopurinol and oxypurinol in rat plasma, intestinal

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wash and bile. Liquid chromatography (LC) with UV detection has been widely used in the determination of allopurinol and oxypurinol concentrations in human plasma and serum [5–7], but the sensitivity reported was inadequate for the drug levels and sample amounts encountered in our study.

Both allopurinol and oxypurinol have been reported to be active on glassy carbon electrodes [7,8]. However, the maximum response for allopurinol is obtained when applied potentials are above +1.2 V. At such high potentials, both sensitivity and selectivity of the oxidative electrochemical detection drastically decline and samples may require an extensive clean-up to obtain the desired sensitivity.

There have been reports using an immobilized xanthine oxidase reactor for highly selective LC detection of xanthine and hypoxanthine [9,10]. This reactor, however, has never been utilized for detection of allopurinol, which is also a substrate for xanthine oxidase. In the published reports, immobilized xanthine oxidase was packed manually into stainless steel or PTFE tubing. The procedure for preparation of the packing included a reaction of aminopropyl glass beads with glutaraldehyde, followed by Schiff's reaction with the enzyme. The procedure appears to be too tedious and time-consuming for routine applications.

In the present study, we have developed an LC method for the simultaneous determination of allopurinol and oxypurinol in rat bile, plasma and intestinal wash. Simultaneous analysis of allopurinol and oxypurinol is achieved by using the post-column xanthine oxidase reactor with the eluent as the reaction medium. The enzyme is immobilized using commercially available aldehyde silica cartridges. Following separation, allopurinol is converted to oxypurinol in a highly specific enzymatic reaction. Oxypurinol is then monitored with high selectivity and sensitivity using oxidative electrochemical detection at a relatively low applied potential (+0.85 V).

The assay is suitable for rapid and sensitive determination of allopurinol and oxypurinol in rat bile, plasma and intestinal wash, allowing an accurate estimation of the rate of absorption of allopurinol from different sites of the gastrointestinal tract.

EXPERIMENTAL

Materials

Allopurinol, oxypurinol and 1-methylxanthine were purchased from Sigma (St. Louis, MO, U.S.A.). Xanthine oxidase (EC 1.2.3.2, buttermilk, 0.4 U/mg of protein) and other chemicals of HPLC grade were obtained from Fluka (Ronkonkoma, NY, U.S.A.).

Apparatus

The apparatus consisted of a Hitachi L-6000 HPLC pump, a 655-40A autosampler, a Hypersil SAS column (250 mm × 4.6 mm, 5-μm), an IMER immobi-

lized enzyme reactor (2 mm × 10 mm, 5-μm aldehyde silica) (Chrompack, Raritan, NJ, U.S.A.) and a dual LC-4B/17AT amperometric detector equipped with a glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A).

Preparation of the immobilized enzyme reactor

An IMER reactor was connected directly to the outlet of an HPLC pump (short, narrow-bore PTFE tubing was used on the inlet) and a small volume of the enzyme solution (2 ml of 1 mg/ml xanthine oxidase in 0.1 *M* Tris buffer, pH 8.2) was allowed to recirculate through the reactor at 1 ml/min for 10 min.

Chromatographic conditions

The mobile phase was prepared by mixing 50 ml of 1 *M* disodium hydrogenphosphate with 50 ml of 1 *M* sodium acetate and 1.9 l of HPLC-grade water and adjusting pH to 7.5 with phosphoric acid. Prior to use, the mobile phase was filtered by aspirating through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.). The flow-rate was 2 ml/min with the analytical column and post-column reactor maintaned at room temperature. The eluent was monitored using a glassy carbon electrode with the potential set at +0.85 V *vs.* an Ag/AgCl electrode.

Rat plasma and bile preparation

A 50-μl aliquot of rat plasma (bile) was diluted with 50 μl of a 0.5 μg/ml internal standard solution and placed into a Ultrafree-MC filter unit (10 000 nominal molecular weight limit (NMWL), polysulfone PTGC membrane, Millipore). Following 30 min centrifugation at 15 000 *g* on a Microfuge-E centrifuge (Beckman), the filtrate was directly injected into the LC system.

Rat intestinal wash preparation

A 20-μl aliquot of rat intestinal wash (4 ml of 0.9% sodium chloride solution in water, rinsed through the corresponding intestinal segment) was added to 1 ml of 0.5 μg/ml internal standard, mixed and directly analyzed.

RESULTS AND DISCUSSION

As it can be seen from Fig. 1, significant oxidation of allopurinol begins to occur only at potentials above +1.0 V. Background current, which is produced mainly through oxidation of mobile phase constituents and impurities, also increases exponentially at these potentials so that the maximum signal-to-noise ratio for allopurinol cannot be realized (Fig. 2). At the same time, the signal-to-noise ratio for oxypurinol reaches its maximum at +0.85 V where fewer of endogenous compounds in biological fluids are electrochemically active, thus providing a high degree of selectivity.

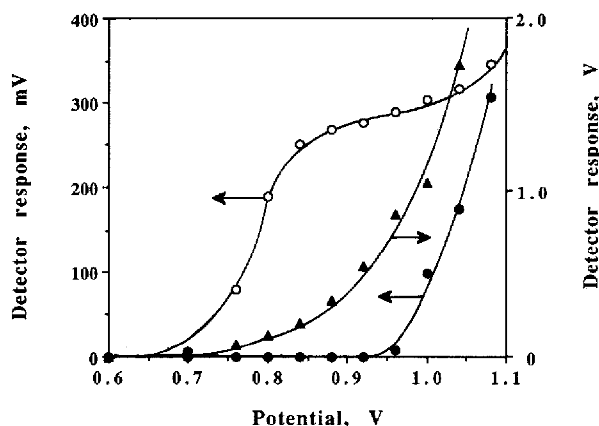
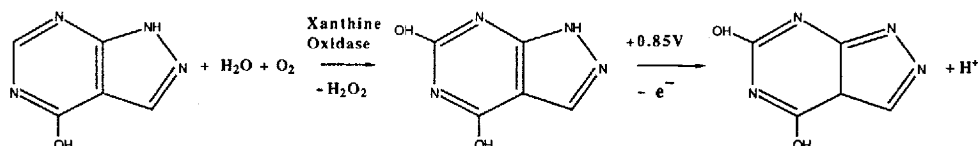


Fig. 1. Hydrodynamic voltagram for allopurinol and oxypurinol, with 75 ng of each compound on-column. Conditions are described in the Experimental section. (○) Oxypurinol; (●) allopurinol; (▲) mobile phase.

The sequence of the post-column enzymatic and electrochemical reactions can be described as follows:



Immobilized xanthine oxidase acts as the "enzyme electrode" mediating electron transfer from allopurinol to an electron acceptor (*e.g.* ferricyanide, cyto-

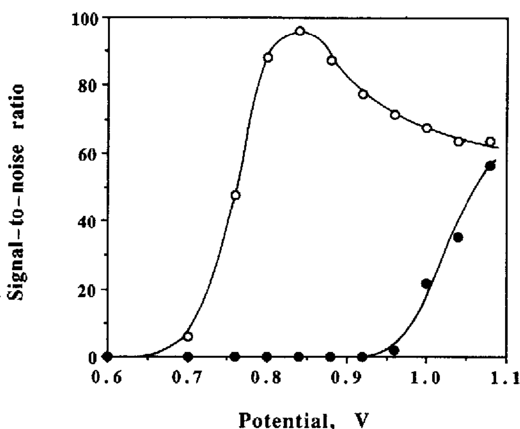


Fig. 2. Signal-to-noise ratio *versus* applied potential for allopurinol and oxypurinol (hydrodynamic conditions), with 75 ng of each compound on-column. Conditions are described in the Experimental section. (○) Oxypurinol; (●) allopurinol.

chrome c or oxygen). If the acceptor is oxygen, the reaction yields oxypurinol and hydrogen peroxide both of which can be detected with high sensitivity. Hydrogen peroxide can be detected electrochemically [11,12] at a platinum electrode or fluorimetrically with more elaborate detection schemes (*e.g.* peroxidase, *p*-hydroxyacetic acid [10]). However, although oxypurinol exhibits xanthine oxidase-inhibiting properties similar to those of allopurinol, it is not a substrate for the enzyme and, therefore, cannot be detected with allopurinol via hydrogen peroxide production. At the same time, both oxypurinol formed from allopurinol in the immobilized enzyme reaction, and metabolic oxypurinol already present in plasma, can be detected simultaneously following separation, via electrochemical oxidation. Oxypurinol is oxidized electrochemically at relatively low potentials with release of an electron to give a 4,7-dehydropyrazolo derivative. Oxidative electochemical detection is not only highly selective at sufficiently low applied potentials, but also does not require extensive deoxygenation of mobile phase, and allows the detection of both allopurinol converted to oxypurinol and the unchanged metabolite with mobile phase as a reaction medium.

For the enzymatic reaction to take place, a sufficient level of an appropriate electron acceptor must be present in the mobile phase. Normally, a large amount of oxygen is dissolved in aqueous buffers (solubility of oxygen in water at 20°C is 1.35 μmol/ml). The concentration of allopurinol in analyzed samples did not exceed 1.8 nmol/ml, with the actual levels in the post-column reactor being much lower, at about 15 pmol/ml (the dilution factor of the analytical column and the reactor was estimated to be 120). The mobile phase, therefore, provided a substantial excess of oxygen over allopurinol in the enzymatic reaction even following degassing during vacuum filtration.

The enzyme activity and/or retention stability did not change significantly over a two-month study period (about 30% decline in the degree of allopurinol to oxypurinol conversion). In the concentration range of allopurinol used, the enzymatic reaction does not follow Michaelis–Menten kinetics, and the reaction rate is independent of allopurinol concentration.

The effect of the flow-rate on degree of conversion of 75 pmol of allopurinol to oxypurinol is shown in Fig. 3. Evidently, the degree of conversion of a substrate depends on the intrinsic rate of the enzymatic reaction and the amount of time at which active sites of the immobilized enzyme in the reactor are exposed to the substrate. This "contact time", in turn, depends on both the rate of diffusion of the substrate molecules through the pores of a support and eluent flow-rate. Apparently, at flow-rates below 1 ml/min, the rate-limiting step for conversion is the diffusion of subtrate molecules through the pores of silica, which is constant for given conditions (support material, temperature, mobile phase viscosity, etc.). At flow-rates from 1 to 3 ml/min, the contributions from diffusion decreases and the eluent flow-rate becomes the rate-limiting step. From Fig. 3, it can be seen that the degree of conversion appears to be approaching a plateau at eluent flow-rates above 3.5 ml/min. One can expect that with diffusion playing an in-

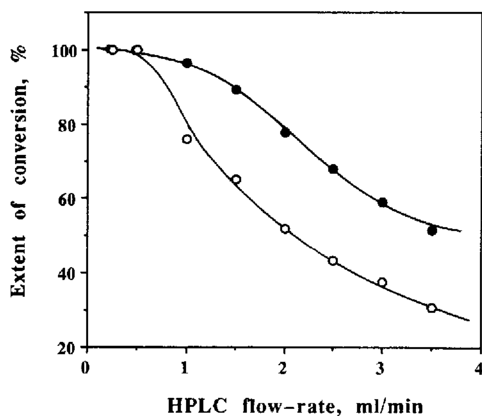


Fig. 3. Effect to the flow-rate on the degree of conversion of allopurinol to oxypurinol and 1-methylxanthine (internal standard) to 1-methyluric acid by immobilized xanthine oxidase. (○) Oxypurinol; (●) allopurinol. The immobilized enzyme reactor is installed between the injector and the analytical column, and the analytes are monitored by a UV detector set at 260 nm. Other conditions are as described in the Experimental section.

creasingly minor role in the total reaction rate, compared to other processes, the rate of conversion will again become independent of flow-rate, depending only on enzyme concentration on outer surfaces of silica particles and the intrinsic enzymatic reaction rate.

Representative chromatograms of a drug-free plasma sample and samples spiked with allopurinol, oxypurinol and 1-methylxanthine as an internal standard are shown in Fig. 4A and B, respectively. Fig. 4C displays a chromatogram of the same sample as in Fig. 4B but with the immobilized enzyme reactor removed. Fig. 4D demonstrates a typical chromatogram of a plasma sample from a rat 10 min following intravenous administration of 1 mg/kg allopurinol. The chromatograms are clean and free of interfering peaks even after minimal clean-up. Of two extraneous peaks appearing at 2.4 and 6.9 min, the first one was identified as endogenous uric acid. When the reactor is removed (Fig. 4C), no allopurinol can be detected.

Calibration

The working standard was diluted serially to provide a standard curve for the concentration range 15.6–500 ng/ml (n = 6) for both allopurinol and oxypurinol. Peak-height ratios of drug to internal standard were plotted *versus* drug concentrations. The slope and intercept of the standard curve were estimated by least-squares linear regression. The linear regression parameters (correlation coefficients, slopes and y-intercepts) exhibited low coefficients of variation (C.V.) over the three-day validation period. The calibration curves were linear over the range with the correlation coefficients exceeding 0.999 for both analytes. The detection

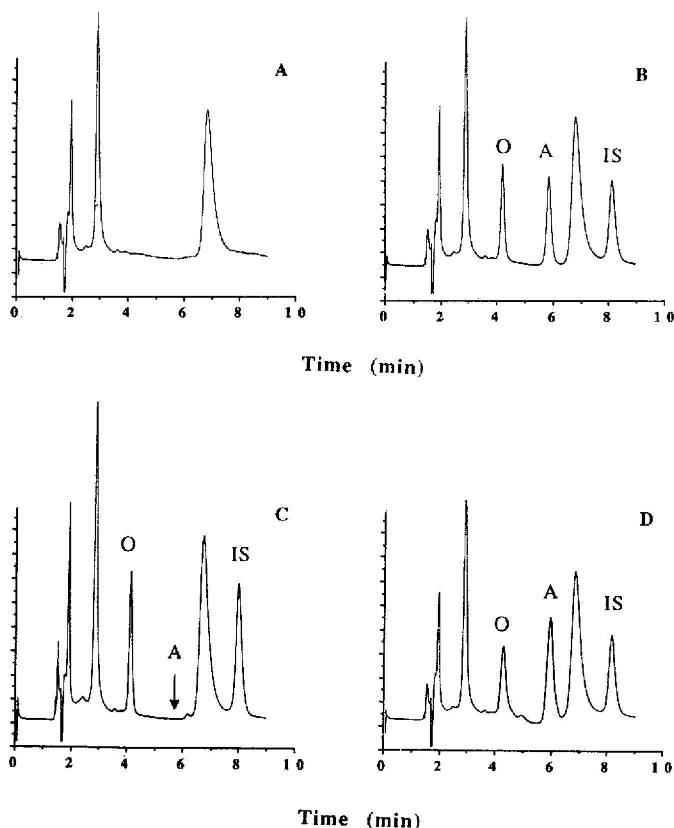


Fig. 4. Chromatograms of rat plasma samples spiked with 0.5 μg/ml 1-methylxanthine (internal standard). Conditions are as described in the Experimental section. (A) Drug-free plasma; (B) plasma spiked with allopurinol and oxypurinol (125 ng/ml); (C) plasma sample as in (B) with the immobilized xanthine oxidase reactor removed; (D) plasma sample 10 min after intravenous administration of 1 mg/kg allopurinol to rat. Peaks: A = allopurinol; O = oxypurinol; IS = internal standard.

limit for both allopurinol and oxypurinol was 31.2 pg on-column, which corresponded to 0.8 ng/ml plasma, at +0.85 V (signal-to-noise ratio = 4).

Reproducibility and recovery

Since the plasma protein binding of allopurinol and oxypurinol has been reported to be quite insignificant (<2%), the concentration of the analytes was not significantly affected by ultrafiltration. To determine the recovery, two rat plasma, intestinal wash and bile pools were prepared at concentrations of allopurinol and oxypurinol of 62.5 and 250 ng/ml each, replicate samples (n = 3) were processed as described in the Experimental section and compared to concentrations of the aqueous standards. All recoveries were higher than 90%. Since the recoveries, linearity ranges and chromatograms of allopurinol and oxypurinol in

TABLE I

ACCURACY AND PRECISION DATA FOR THE DETERMINATION OF ALLOPURINOL AND OXYPURINOL IN RAT PLASMA USING THE IMMOBILIZED XANTHINE OXIDASE REACTOR

The within-day precision, between-day precision and accuracy are defined in the text.

Compound	Plasma concentration (ng/ml)	Assay concentration (mean ± S.D.) (ng/ml)	*F*-ratio[a]	Within-day precision ($n=5$) (%)	Between-day precision ($n=15$) (%)	Accuracy (mean ± S.D.) ($n=3$)
Oxypurinol	250.0	249.4 ± 3.9	0.81	1.6	2.0	0.99 ± 0.02
	62.5	61.3 ± 1.3	2.13	2.1	7.5	0.98 ± 0.02
Allopurinol	250.0	246.5 ± 2.3	0.09	0.9	1.0	0.99 ± 0.01
	62.5	62.6 ± 1.5	3.65	2.43	3.4	1.00 ± 0.02

[a] The critical *F*-value for the between-day variance was 3.59 (two degrees of freedom) at a significance level of 0.05.

plasma do not differ significantly from those in intestinal wash and bile, the method was validated by performing replicate analyses ($n = 5$) of spiked rat plasma pools (62.5 and 250 ng/ml allopurinol and oxypurinol) on three separate days.

Concentrations were determined by comparison with a standard curve prepared on the day of analysis. The precision and overall accuracy of the method were estimated (Table I). The within-day precision, expressed as the mean of the daily C.V., for both compounds ranged from 0.9 to 2.5%. Analysis of variance (ANOVA) tests indicated no significant between-day variation (Table I) and so data were pooled ($n=15$). The between-day precision observed for both compounds, and defined as the C.V. of the pooled data at each concentration level, was in the range 1.0–7.5%. The accuracy of the method expressed as the ratio of predicted to actual concentration (*C* ratio) was in the range 0.98–1.00.

The method has several advantages over previously described techniques: the enzyme immobilization is extremely simple, since an activated silica reactor is commercially available. High selectivity of the detection scheme yields sensitivity about 50 times higher than previously reported for allopurinol.

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