September 1989

Chem. Pharm. Bull. 37(9) 2459—2462 (1989)

Simultaneous Assay of Hypoxanthine, Xanthine and Allopurinol by High-Performance Liquid Chromatography and Activation of Immobilized Xanthine Oxidase as an Enzyme Reactor

Masahiro KITO,* Riichi TAWA, Shigeo TAKESHIMA and Shingo HIROSE

Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607, Japan. *Received February 13, 1989*

A selective and sensitive assay of substrates (hypoxanthine, xanthine and allopurinol) of xanthine oxidase by reversed-phase liquid chromatography coupled with the use of immobilized enzyme reactors is described. These compounds were oxidized by immobilized xanthine oxidase and produced hydrogen peroxide, which was determined fluorometrically using immobilized peroxidase and *p*-hydroxyphenylacetic acid. The detection limits of hypoxanthine, xanthine and allopurinol were approximately 50, 120 and 130 pg per injection, respectively.

Immobilized xanthine oxidase inhibited by oxipurinol during the assay was reactivated by 2,6-dichlorophenolindophenol and could be used for a long period without a significant activity loss. These methods were applied to plasma and urine samples.

Keywords HPLC; hypoxanthine; xanthine; allopurinol; reactivation; xanthine oxidase; 2,6-dichlorophenolindophenol; fluorometry; immobilized enzyme reactor

Allopurinol(4-hydroxypyrazolo[3,4-*d*]pyrimidine, Allo), widely used in the treatment of gout and other hyperuricemic states,[1] is an alternate-substrate inhibitor of xanthine oxidase and is metabolized mainly to oxipurinol, which is also a potent inhibitor of xanthine oxidase. Administration of Allo results in a decrease in uric acid and an increase in hypoxanthine (Hyp) and xanthine (Xan) in serum and urine. Therefore, the simultaneous determination of Hyp, Xan and Allo in biological fluids is required for studies of the pharmacological effects of Allo in the clinical field.

Recently, high-performance liquid chromatography (HPLC) has become popular for the assay of these compounds.[2] Most of the HPLC methods are dependent on ultraviolet absorption measurement, which is often unsatisfactory for sensitive and selective assay due to many interfering compounds. On the other hand, the enzyme binding assay for Allo and oxipurinol does not permit the simultaneous assay of Hyp, Xan and Allo.[3]

We have already reported the assay of Hyp and Xan by HPLC coupled with enzyme reactors; after separation by HPLC, hydrogen peroxide produced from Hyp and Xan by immobilized xanthine oxidase (IXO) was determined fluorometrically using immobilized peroxidase (IPO) and *p*-hydroxyphenylacetic acid.[4] With this method, it is also possible to measure Allo, which can be converted by IXO to oxipurinol and hydrogen peroxide.

However, a large number of samples (above 40) containing Allo could not be analyzed accurately, due to the tight-binding inhibition of IXO by the major metabolite oxipurinol.[5] This inhibition can be reversed by treatment with electron acceptors, such as 2,6-dichlorophenolindophenol, phenazine methosulfate, potassium ferricyanide or methylene blue.[6]

In this report, we describe a method for reactivation of the inhibited IXO and a sensitive and an accurate procedure for simultaneous determination of Hyp, Xan and Allo in plasma or urine by HPLC coupled with the enzyme reactors.

Experimental

Chemicals Standard solutions of Hyp, Xan and Allo (Nakarai Chemicals, Kyoto, Japan) were prepared by dissolving them in minimum amounts of potassium hydroxide solution and diluting the solutions with 0.01 mol/l phosphate buffer (pH 5.5) before use. *p*-Hydroxyphenylacetic acid (Nakarai Chemicals) was purified by sublimation and dissolved in 0.1 mol/l phosphate buffer (pH 10.5) before use. Potassium ferricyanide, phenazine methosulfate and 2,4-dichlorophenolindophenol sodium salt (Nakarai Chemicals) were dissolved in 0.1 mol/l phosphate buffer (pH 7). Unless otherwise stated all reagents were of analytical-grade quality.

Preparation of the Enzyme Reactors The immobilizations of xanthine oxidase (cow's milk, 0.4 unit/mg protein, Boehringer-Mannheim Yamanouchi, Tokyo, Japan) and peroxidase (horseradish, 264 units/mg protein, Toyobo, Tokyo, Japan) on aminopropyl controlled-pore glass (80—530 Å mean pore diameter, Electro-Nucleonics, Fairfield, U.S.A.) were reported in detail previously.[4,7] IXO and IPO were placed in stainless-steel or PTFE tubes. Immobilized enzymes in the reactors used were kept in 0.1 mol/l phosphate buffer (pH 7) at 4 °C.

Assay Procedure A diagram of the HPLC system coupled with the enzyme reactors and the reaction schemes[8] employed in the post-column reactors used in this study are shown in Fig. 1. The HPLC system used was the same as that used previously.[4]

A guard column (1 × 0.4 cm) and an analytical column (20 × 0.4 cm) were prepared with Nucleosil C18 (5 μm, Macherey, Nagel & Co., Duren, F.R.G.) using the usual technique. The separations were carried out by eluting with 0.01 mol/l phosphate buffer (pH 5.5) containing 1% (v/v) acetonitrile at a flow rate of 0.7 ml/min.

IXO and IPO were placed in stainless-steel tubes, 5 × 0.2 cm and 15 × 0.2 cm, respectively. The effluent from the analytical column was adjusted to pH 7.7 by adding 0.1 mol/l phosphate buffer (pH 10.5) with pump 2 (flow rate of 0.3 ml/min) (Hyp, Xan and Allo were oxidized to yield hydrogen peroxide in the IXO reactor). The effluent from the IXO reactor was adjusted to pH 8.2 by adding 0.1 mol/l phosphate buffer (pH

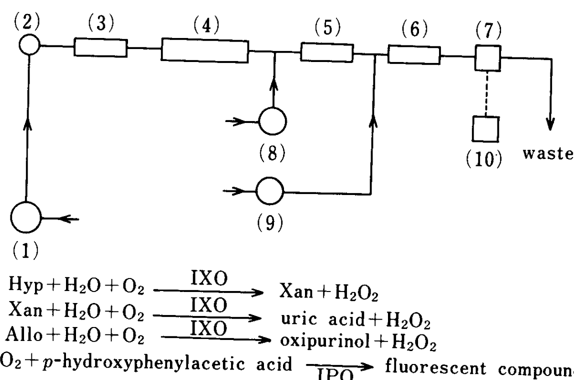

Fig. 1. Flow Diagram of HPLC Coupled with the Immobilized Enzyme Reactors and the Post Column Reactions

(1) HPLC pump, (2) sample injector, (3) guard column, (4) analytical column, (5) IXO reactor, (6) IPO reactor, (7) fluorescence detector, (8) pump 2, (9) pump 3, (10) integrator.

© 1989 Pharmaceutical Society of Japan

NII-Electronic Library Service

10.5) containing 1.8 mmol/l *p*-hydroxyphenylacetic acid with pump 3 (flow rate of 0.4 ml/min). *p*-Hydroxyphenylacetic acid was oxidized in the IPO reactor by hydrogen peroxide produced from Hyp, Xan and Allo, to form a highly fluorescent compound (excitation maximum 320 nm, emission maximum 407 nm). Plasma and urine samples were prepared as described previously.[4] A mixture of 0.5 ml of plasma and 6% (w/v) trichloroacetic acid solution was centrifuged for 15 min at 1500 g. An appropriate volume of the supernatant was added to an equal volume of 0.5 mol/l tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane. After mixing and centrifuging, 10 or 20 μl of the aqueous solution was injected into the analytical system. Urine was heated for 10 min at 70 °C, mixed with an equal volume of 1 mol/l phosphate buffer (pH 9) and filtered. A 0.5 ml aliquot of this solution was diluted with 4.5 ml of 0.01 mol/l phosphate buffer (pH 5) and a sample was injected (5 μl).

Determination of the Activities of IXO A 10 μl aliquot of 0.5 mmol/l Allo or Xan was injected into the flow system consisting of a peristaltic pump (model MP-3, Tokyo Rica, Japan), a sample injector (model SIL-1A, Shimadzu, Japan) and the IXO reactor (4 × 0.1 cm; PTFE tube). The experiments were carried out in air without any special treatment. The effluents containing Allo and oxipurinol from the flow system were collected and determined by HPLC (model LC-3A, Shimadzu) equipped with an ultraviolet detector (254 nm) (model UVD-1, Shimadzu). The column (Nucleosil C18, 5 × 0.4 cm) was eluted with 0.01 mol/l phosphate buffer (pH 5.5) at the flow rate of 1 ml/min. The relative activity of the IXO reactor was calculated on the basis of the original activity.

Results and Discussion

Inhibition and Reactivation of IXO The inhibition of xanthine oxidase by Allo appears to occur chiefly through its major metabolite oxipurinol, which forms a tightly bound complex with reduced xanthine oxidase under anaerobic conditions[5] (Fig. 2). Therefore, it can be considered that IXO in the reactor is inhibited only when reduced IXO is formed in the presence of oxipurinol. In practice, IXO was not inhibited by oxipurinol contained in the sample, but was inhibited by oxipurinol produced from Allo in the IXO reactor. Slow deactivation of IXO (reactor size of 5 × 0.2 cm) used in the assay was observed after 40 reactions with 0.5 mmol/l of Allo. However, the relative activity of IXO (reactor size of 4 × 0.1 cm) was remarkably decreased with increasing number of exposures to Allo (Fig. 3). IXO was not inhibited by Hyp, Xan or uric acid at concentrations as low as 5×10^{-3} mol/l.

Mild oxidants, such as 2,6-dichlorophenolindophenol, phenazine methosulfate and potassium ferricyanide were used in the present studies of reactivation of IXO bound with oxipurinol. These oxidants have often been used in the reoxidation of reduced xanthine oxidase. Each oxidant efficiently reactivated IXO inhibited by Allo, as shown in Fig. 3. The effect of oxidants on the enzyme reaction was also studied. Both potassium ferricyanide and 2,6-dichlorophenolindophenol at concentrations as low as 10^{-5} mol/l did not influence the IXO or IPO reaction. On the other hand, phenazine methosulfate reacted with *p*-hydroxyphenylacetic acid in the IPO reactor and formed a fluorescent compound interfering with sensitive measurements of Hyp, Xan and Allo.

Reactivation efficiencies are shown in Fig. 4. A longer time (about 40 min) was required for full reactivation by 5×10^{-4} mol/l potassium ferricyanide, while 20 min was enough when 5×10^{-3} mol/l was used. Full reactivation by 5×10^{-4} mol/l 2,6-dichlorophenolindophenol was achieved within 10 min.

The following treatments were, therefore, used for reactivation of IXO used in the present assay: the IXO reactor was washed with 5×10^{-4} mol/l 2,6-dichloroin-

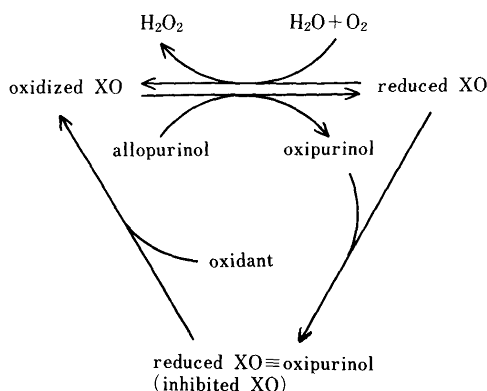

Fig. 2. Schematic Representation of the Inhibition of XO by Allo

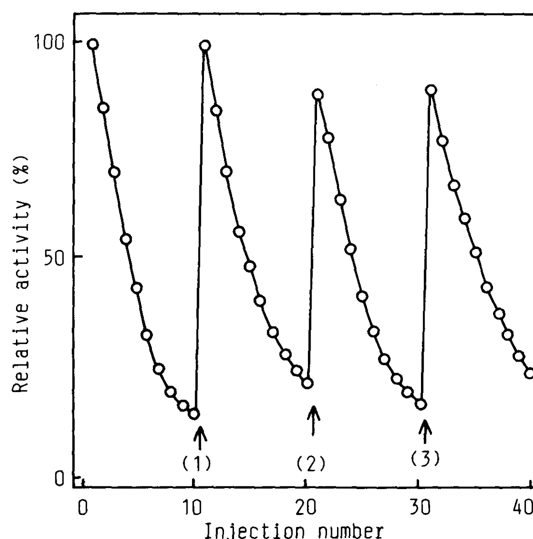

Fig. 3. Inhibition by Allo and Reactivation by Oxidants

A 10 μl aliquot of 0.5 mmol/l Allo was injected repeatedly into the IXO reactor (4 × 0.1 cm). A solution containing 5×10^{-4} mol/l of 2,4-dichlorophenolindophenol (1), potassium ferricyanide (2) or phenazine methosulfate (3) was flushed through the IXO reactor with the peristaltic pump (10 min) after every 10 injections of Allo.

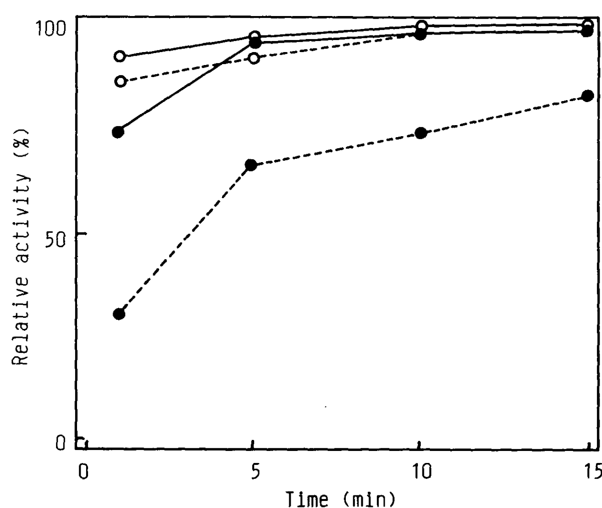

Fig. 4. Reactivation of Inhibited IXO by 2,6-Dichlorophenolindophenol (—○—, 5×10^{-4} mol/l; ---○---, 5×10^{-5} mol/l) and Potassium Ferricyanide (—●—, 5×10^{-3} mol/l; ---●---, 5×10^{-4} mol/l)

Oxidants were flushed through the inhibited IXO for various lengths of time by the peristaltic pump (flow rate of 0.2 ml/min) and then activities were determined by measuring the conversion of Xan to uric acid.

NII-Electronic Library Service

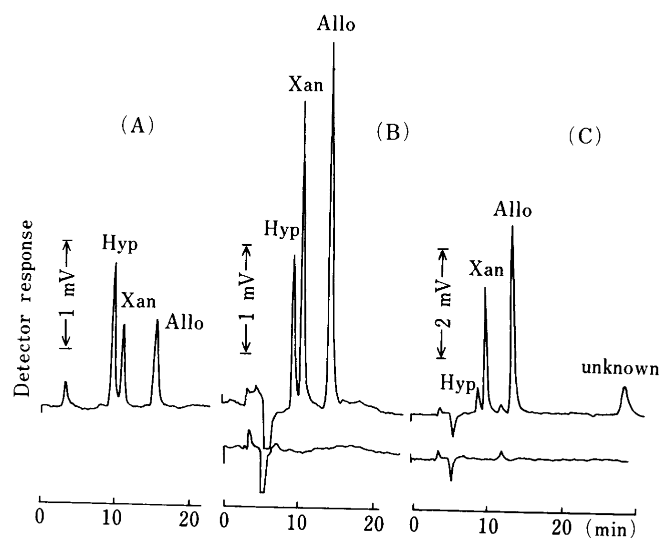

Fig. 5. Chromatograms of (A) Standard Mixture (1 μmol/l), (B) Plasma Sample and (C) Urine Sample Obtained by the Present Method (Upper), and Obtained when the IXO Reactor was Removed from the System (Lower)

Biological samples were collected from a volunteer at 2 h after the oral administration of 2.8 mg/kg of Allo. Injection volumes of standard, plasma and urine samples were 10, 20 and 5 μl, respectively.

dophenol for 10 min and then washed with 0.1 mol/l phosphate buffer (pH 7) for 5 min. The latter treatment removed oxidant in the reactor. When this procedure was employed, the IXO reactor could be used in the sensitive and accurate determination of Hyp, Xan and Allo in biological samples over a long period.

Chromatograms of Biological Fluids The chromatograms of plasma sample taken from a volunteer at 2 h after the oral administration of 2.8 mg/kg of Allo are shown in Fig. 5B. The upper chromatogram was obtained by using the system illustrated in Fig. 1 and the lower chromatogram was obtained when the IXO reactor was removed. No peak appeared after elution of Allo. These results show that the selective assay of Hyp, Xan and Allo in plasma can be performed within 15 min and that there are no interfering peaks in the areas where Hyp, Xan and Allo elute. A dip in the base-line at 7 min was due to the quenching of the blank fluorescence by uric acid existing in plasma at high concentrations.

The assay of urine samples required a longer time than the assay of plasma samples because of elution of an unknown compound at 28 min which reacted with IXO (Fig. 5C).

Determination of Hyp, Xan and Allo The calibration curves of Hyp, Xan and Allo were linear over the concentration range from 0.5 to 10 μmol/l with intercepts not significantly different from zero (the correlation coefficients were 0.999). The limits of determination for Hyp, Xan and Allo were approximately 50, 120 and 130 pg per injection, respectively.

The intra-assay (within-day) CV_s, which were estimated by repeated determinations ($n=14$) of a standard mixture (10 μl) containing 1 μmol/l of Hyp, Xan and Allo, were 4.0, 5.1 and 5.9%, respectively. The inter-assay (between-day) CV_s on different six days were 8.6% for Hyp, 5.6% for Xan and 10.6% for Allo. The intra-assay CV_s were within acceptable limits, but the inter-assay CV_s were slightly high.

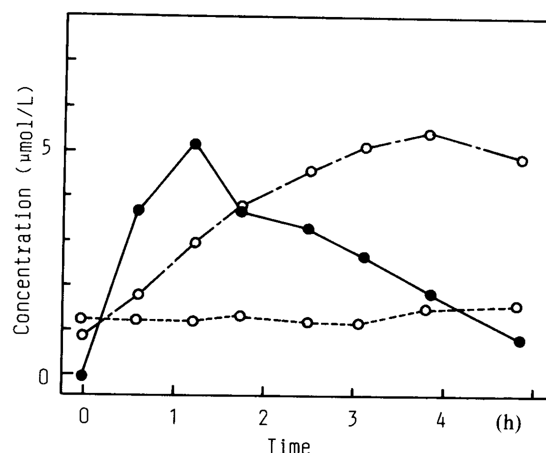

Fig. 6. Plasma Concentration–Time Curves of Hyp (---○---), Xan (--○--) and Allo (—●—) in a Volunteer Subject after Oral Administration of Allo (2.8 mg/kg)

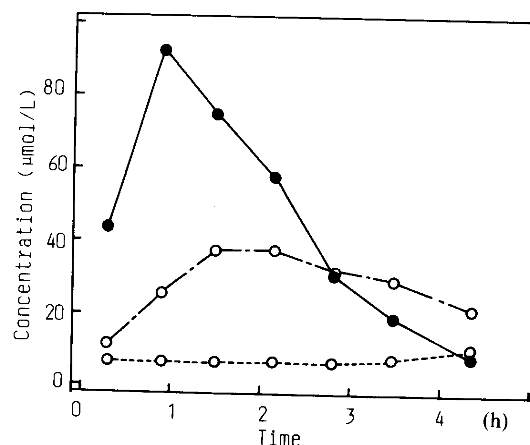

Fig. 7. Urine Concentration–Time Curves of Hyp, Xan and Allo in a Volunteer Subject after Oral Administration of Allo (2.8 mg/kg)

Urine was collected at 0—0.6, 0.6—1.2, 1.2—1.8, 1.8—2.5, 2.5—3.1, 3.1—3.9 and 3.9—4.9 h after administration of Allo. The x values are in the middle of each time period. Other conditions were as in Fig. 6.

Therefore, the concentrations of Hyp, Xan and Allo in biological samples were determined using calibration curves prepared by measurement of three concentrations on each day.

Analytical recoveries from the control serum and urine samples added to yield final concentrations of 2 μmol/l ($n=4$) were 93—101% for Hyp, Xan and Allo.

Representative plots of concentrations of these compounds in plasma *vs.* time for a volunteer subject after a single oral administration of Allo (2.8 mg/kg) are shown in Fig. 6. Analytical values of Xan in plasma were increased by Allo's inhibition of xanthine oxidase. However, the values of Hyp were not changed within 5 h after a single oral administration. Rabbit liver aldehyde oxidase can catalyze the conversion of Hyp to Xan as well as Allo to oxipurinol, like xanthine oxidase, but not the conversion of Xan to uric acid.[9] Therefore, it can be considered that human aldehyde oxidase might also oxidize only Hyp, like the rabbit enzyme. Also, urine samples showed almost the same behavior as plasma samples (Fig. 7). Potter and Silvidi reported that the Xan concentration in serum and urine of a leukemia patient treated with Allo increased to

NII-Electronic Library Service

extremely high levels; such elevated Xan levels led to the formation of stones, which contained 82% Xan, 15% oxipurinol and 3% Hyp, in the kidneys.[10] Measurement of Allo and Xan in patients treated with Allo would be useful for prevention of formation of such stones.

In this study, the analytical column was used for about 400 applications. The guard column was renewed after every 40 applications. The IXO reactor (which was reactivated after every 40 applications) and the IPO reactor were used for about 200 applications. Unfortunately, it is impossible to determine oxipurinol with this method, but the effect of Allo treatment on Hyp and Xan levels in biological fluids can be easily monitored.

References

1) T. F. Yu and A. B. Gutman, *Am. J. Med.*, **37**, 885 (1964); G. B. Elion, A. Kovensky and G. H. Hitchings, *Biochem. Phamacol.*, **15**, 863 (1966); G. B. Elion, S. Callahan, H. Nathan, S. Bieber, R. W. Rundless and G. H. Hitchings, *ibid.*, **12**, 85 (1963).
2) A. Mcburney and T. Gibson, *Clin. Chim. Acta*, **102**, 19 (1980); W. E. Wung and S. B. Howell, *Clin. Chem.*, **26**, 1704 (1980); H. Miyazaki, Y. Matsunaga, K. Yoshida, S. Arakawa and M. Hashimoto, *J. Chromatogr.*, **274**, 75 (1983); R. Boulieu, C. Bory and P. Baltassat, *ibid.*, **307**, 469 (1984); T. Kojima, T. Nishina, M. Kitamura, T. Hosoya and K. Nishioka, *Clin. Chim. Acta*, **137** 189 (1984).
3) K. R. Hande and B. A. Chabner, *Anal. Biochem.*, **101**, 26 (1980).
4) M. Kito, R. Tawa, S. Takeshima and S. Hirose, *J. Chromatogr.*, **278**, 35 (1983).
5) T. Spector and D. G. Johns, *J. Biol.Chem.*, **245**, 5079 (1970); S. Cha, R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmacol.*, **24**, 2187 (1975); T. Spector, *ibid.*, **26**, 355 (1977).
6) T. Spector and D. G. Johns, *Biochem. Biophys. Res. Commun.*, **32**, 1039 (1968).
7) R. Tawa, M. Kito, S. Hirose and K. Adachi, *Chem. Pharm. Bull.*, **30**, 615 (1982).
8) G. G. Guilbault, P. Brignac, Jr. and M. Zimmer, *Anal. Chem.*, **40**, 190 (1968).
9) T. A. Krenitsky, S. M. Neil, G. B. Elion and G. H. Hitchings, *Arch. Biochem. Biophys.*, **150**, 585 (1972).
10) J. L. Potter and A. A. Silvidi, *Clin. Chem.*, **33**, 2314 (1987).

NII-Electronic Library Service