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FLUOROMETRIC DETERMINATION OF HYPOXANTHINE AND XANTHINE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ENZYME REACTORS

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SUMMARY

A selective and sensitive assay of hypoxanthine and xanthine in biological fluids by high-performance liquid chromatography coupled with immobilized-enzyme reactors was developed. The separations were achieved by reversed-phase liquid chromatography. Hydrogen peroxide produced from hypoxanthine and xanthine by immobilized xanthine oxidase was determined fluorometrically using immobilized peroxidase and *p*-hydroxyphenylacetic acid. Immobilized enzymes were prepared by intermolecular cross-linking to controlled-pore glass. Assay of allopurinol was also possible by the present method. The method was applied to serum and urine. The detection limits of hypoxanthine and xanthine were approximately 50 and 120 pg per injection, respectively.

INTRODUCTION

The determination of hypoxanthine (Hyp), xanthine (Xan), and/or allopurinol, which is a potent xanthine oxidase inhibitor used in the treatment of gout, in biological fluids has been required for various pharmacological and physiological reasons [1—3]. As a part of these studies, we needed a rapid, accurate and sensitive assay for the substrates of xanthine oxidase because we wished to analyze a large number of samples containing various amounts of these compounds.

Recently, high-performance liquid chromatography (HPLC) has become popular for the assay of nucleosides and bases involving Hyp and Xan [4—10]. Unfortunately, such methods were unsuitable for the rapid assay of the compounds of interest. On the other hand, enzymatic methods for the deter-

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mination of Hyp and Xan have been published [11, 12]. However, these methods do not permit the simultaneous assay of Hyp and Xan.

We have already reported a simultaneous and specific determination of Hyp and Xan using HPLC coupled with enzyme reactors [13—15]; uric acid produced from Hyp and Xan by immobilized xanthine oxidase (IXO) was determined at 290 nm. However, we could not determine Xan in normal serum accurately due to the lack of adequate sensitivity and selectivity of this method (the detection limit of Xan was 0.5 μmol/l). Hartwick et al. [4] found Xan in normal serum in the concentration range 0.542—4.7 μmol/l.

In this report, we describe a sensitive fluorometric determination of Hyp and Xan using HPLC coupled with enzyme reactors. The assay employed the following principles as the post-column reactions [16]:

$$\text{Hyp} + H_2O + O_2 \xrightarrow{\text{xanthine oxidase}} \text{Xan} + H_2O_2$$

$$\text{Xan} + H_2O + O_2 \xrightarrow{\text{xanthine oxidase}} \text{uric acid} + H_2O_2$$

$$H_2O_2 + p\text{-hydroxyphenylacetic acid} \xrightarrow{\text{peroxidase}} \text{fluorescent compound}$$

Various substrates of peroxidase have been reported [17]; we chose *p*-hydroxyphenylacetic acid because of the lower cost and the lower fluorescence blank than the others.

The present method may easily be instituted as a routine laboratory procedure in the investigation of purine metabolism.

EXPERIMENTAL

Chemicals

Standard solution of Hyp, Xan, allopurinol (Nakarai Chemicals, Kyoto, Japan) and uric acid (Merck, Darmstadt, F.R.G.) were made by dissolving them in diluted sodium hydroxide and diluting to the desired volume with 0.01 *M* phosphate buffer (pH 5.5) before use. *p*-Hydroxyphenylacetic acid (Nakarai Chemicals) purified by sublimation was dissolved in 0.1 *M* phosphate buffer (pH 10). Enzymes used were xanthine oxidase (cow's milk, 0.4 units/mg protein, Boehringer-Mannheim-Yamanouchi, Tokyo, Japan), peroxidase (horseradish, 264 units/mg protein, Toyobo, Tokyo, Japan) and catalase (bovine liver, 37,000 units/mg protein, Sigma, St. Louis, MO, U.S.A.). An aminopropyl controlled-pore glass (80—530 Å mean pore diameter, Electro-Nucleonics, Fairfield, U.S.A.) was used as a carrier for the immobilization of enzymes. All other reagents were of analytical reagent grade.

Apparatus

The liquid chromatograph used was a Model LC-3A (Shimadzu, Kyoto, Japan). Solutes were monitored with a variable-wavelength ultraviolet (UV) detector Model SPD-2A (Shimadzu) and a fluorescence spectrometer Model RF-500LC (Shimadzu) equipped with an integrator Model C-R1A (Shimadzu). Reagent for the enzymatic reaction was added with a mini-micro pump Model KHD-16 (Kyowaseimitu, Tokyo, Japan).

Preparation of immobilized enzymes

The immobilization procedure for xanthine oxidase and catalase was the same as that reported previously [14]; enzyme was attached to an aminopropyl controlled-pore glass by the intermolecular cross-linking method.

The immobilization of peroxidase was achieved by cleaving its carbohydrate residues with periodic acid followed by coupling the enzyme to aminopropyl controlled-pore glass. A 5-mg portion of peroxidase was dissolved in 2 ml of 0.05 *M* phosphate buffer (pH 7). This solution was mixed well with 1 ml of 5 μM periodic acid in water for 4 h at room temperature, then the mixture was dialyzed overnight against 500 ml of 0.05 *M* phosphate buffer (pH 7). The dialyzed solution was added to 0.5 g of aminopropyl controlled-pore glass and allowed to react at 4°C overnight. The glass beads carrying enzyme were filtered and washed with 100 ml of cold water followed by washing with 100 ml of 1 *M* sodium chloride and 1000 ml of cold water.

IXO and immobilized peroxidase (IPO) were filled into stainless-steel tubes, 5 × 0.21 cm and 15 × 0.21 cm, respectively, for HPLC; PTFE tubes, 5 × 0.1 cm, were used for other experiments.

Chromatographic conditions

A pre-column (1.0 × 0.4 cm) and an analytical column (20 × 0.4 cm) were prepared with Nucleosil 5 C_{18} (5 μm, Macherey, Nagel & Co., Düren, F.R.G.) using a high-pressure slurry-packing technique.

The separations were performed by eluting with 0.01 *M* phosphate buffer (pH 5.5) containing 2% (v/v) acetonitrile at a flow-rate of 0.7 ml/min.

Sample purification

Serum purification was performed by the procedure described by Khym [18]. A 0.5-ml portion of serum was mixed with an equal volume of a 6% (w/v) solution of trichloroacetic acid, and the mixture was centrifuged for 15 min at 1500 *g*. An appropriate volume of the supernatant was added to an equal volume of 0.5 *M* tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane. After mixing and centrifuging, 5 or 10 μl of the aqueous solution were injected into the analytical column.

Recovery experiments were carried out by adding known amounts of Hyp and Xan to control serum. The recoveries of Hyp and Xan were 93 ± 9.9% (n = 4) and 96 ± 9.2% (n = 4), respectively.

Urine purification was performed by the following method. A 1-ml aliquot of urine was heated for 10 min at 70°C and mixed with 1 ml of 1 *M* phosphate buffer (pH 9) and filtered; 0.5 ml of this solution was adjusted to neutral pH by adding 4.5 ml of 0.01 *M* phosphate buffer (pH 5). A 10-μl volume of this solution was injected. Recoveries were approximately 100%.

RESULTS AND DISCUSSION

Reactor properties

The properties and the conversion efficiencies of IXO and IPO were calculated from UV-absorptiometric and fluorometric determinations, using a flow-injection method. The pH optima of IXO and IPO were confirmed

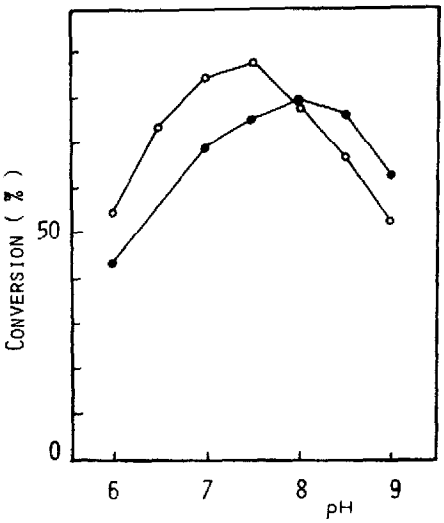

Fig. 1. Effects of pH on the conversion efficiency of IXO (○) and IPO (●).

as 7.5 and 8.0, respectively (Fig. 1). The conversion efficiencies as a function of the amount of acetonitrile in the solvent were studied. Acetonitrile contents lower than 3% (v/v) did not degrade the activities of either immobilized enzyme. The effect of temperature on conversion efficiencies was also studied and there was no significant difference between optimum (37°C) and room (25°C) temperature. Both immobilized enzymes retained their performance without decrease in activity for more than a month.

Inclusion of immobilized-enzyme reactors into the chromatographic system

A diagram of the HPLC system coupled with enzyme reactors used in this study is shown in Fig. 2. After separation on the analytical column, the effluent was adjusted to pH 7.5 by adding 0.1 *M* phosphate buffer (pH 10) with pump 2 at a flow-rate of 0.35 ml/min; Hyp and Xan were oxidized to hydrogen peroxide and uric acid in the IXO reactor. The effluent from the IXO reactor was adjusted to pH 8.0 by adding 0.1 *M* phosphate buffer (pH 10) containing 1.6 m*M* *p*-hydroxyphenylacetic acid with pump 3 at a flow-rate of 0.46 ml/min; *p*-hydroxyphenylacetic acid was oxidized by hydrogen

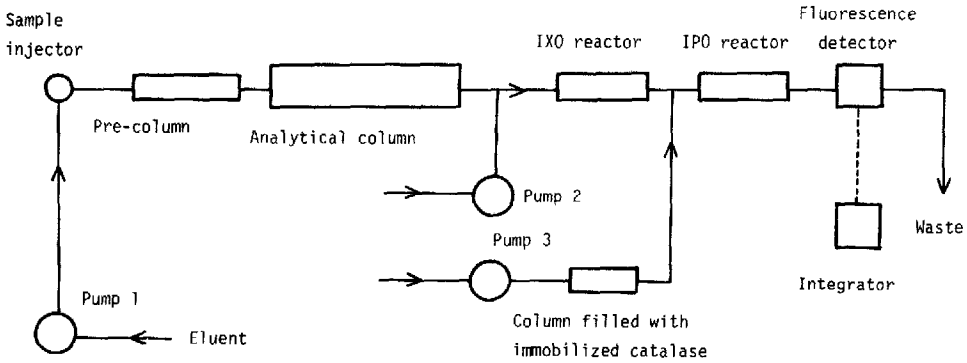

Fig. 2. Flow diagram of HPLC coupled with the immobilzed-enzyme reactors.

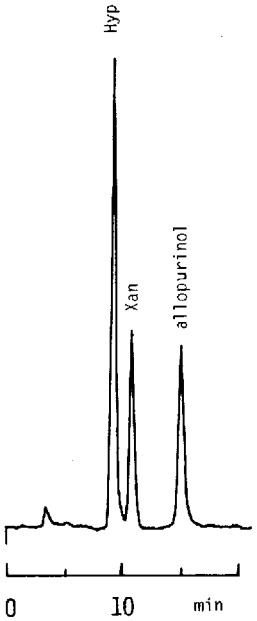

Fig. 3. Chromatogram of a mixture containing 400 ng/ml each of uric acid, Hyp, Xan and allopurinol obtained using the present method. Column: Nucleosil 5 C_{18}, 20 × 0.4 cm. Eluent: 0.01 *M* phosphate buffer (pH 5.5) containing 2% (v/v) acetonitrile. Flow-rates from pump 1, pump 2 and pump 3: 0.7, 0.34 and 0.45 ml/min, respectively. Injection volume: 10 μl. Fluorescence detection: excitation 320 nm, emission 407 nm.

peroxide, produced from Hyp and Xan, in the IPO reactor to form a highly fluorescent compound, which has an excitation maximum of 320 nm and an emission maximum of 407 nm. Then, the column (1.0 × 0.4 cm) filled with immobilized catalase was used to reduce the blank fluorescence in the solution containing *p*-hydroxyphenylacetic acid.

Fig. 3 shows the chromatogram of a standard solution containing uric acid, Hyp, Xan and allopurinol using the present system. There was a little peak broadening in the reactors.

Chromatograms of biological fluids

Fig. 4. shows chromatograms of a serum extract from a normal subject. Chromatogram A was obtained using the system illustrated in Fig. 2 and chromatogram B was obtained when the IXO reactor was removed. These show that the selective assay of Hyp and Xan is performed within 15 min and that the peaks of both Hyp and Xan are eluted free from other fluorescent compounds. Chromatogram C was obtained at an UV absorbance of 254 nm when all the reactors had been removed and shows that the simultaneous quantitative determination of Hyp and Xan is impossible.

Fig. 5 shows chromatograms of urine from a normal subject. The compound eluting at 33 min which reacts with xanthine oxidase to produce hydrogen peroxide, was not determined in this study.

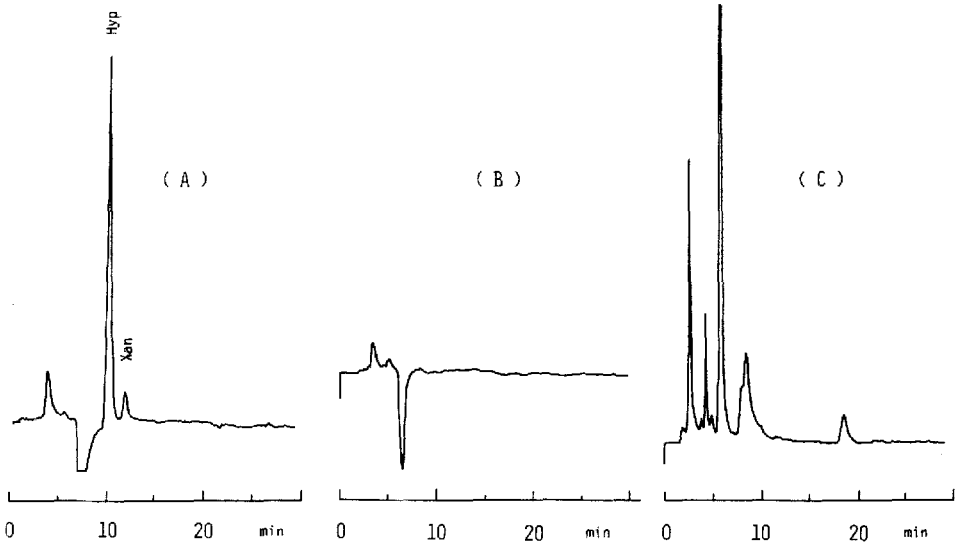

Fig. 4. Chromatograms of a serum extract from a normal subject obtained by the present method (A), when the IXO reactor is removed from the system (B), and with UV absorbance at 254 nm when all reactors are removed (C). Chromatographic conditions as in Fig. 3.

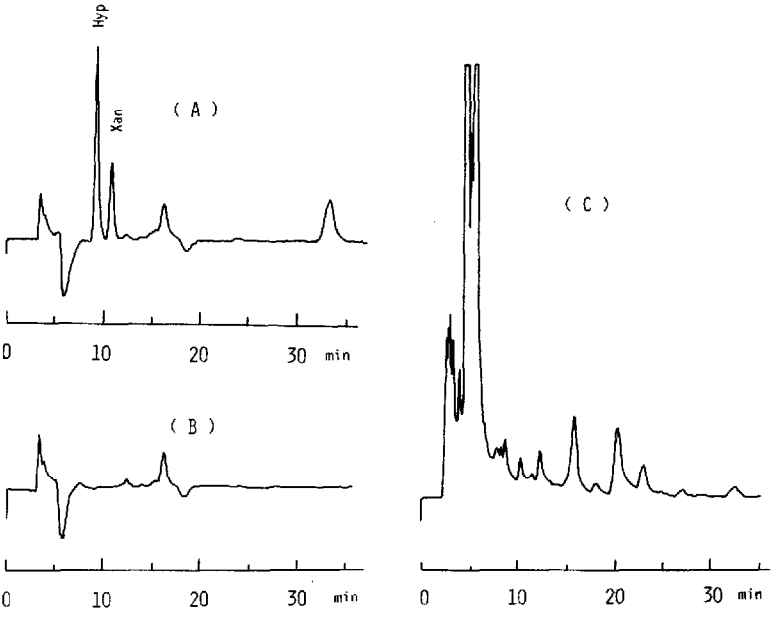

Fig. 5. Chromatograms of urine from a normal subject. Conditions as in Fig. 4.

Analysis of Hyp and Xan

The linearity of calibration curves of concentration versus peak area was excellent for both Hyp and Xan, ranging from 0.74 to 13.15 μmol/l. The average coefficients of variation for the normalized peak area over this range of Hyp and Xan concentrations were 2.87 and 3.48%, respectively.

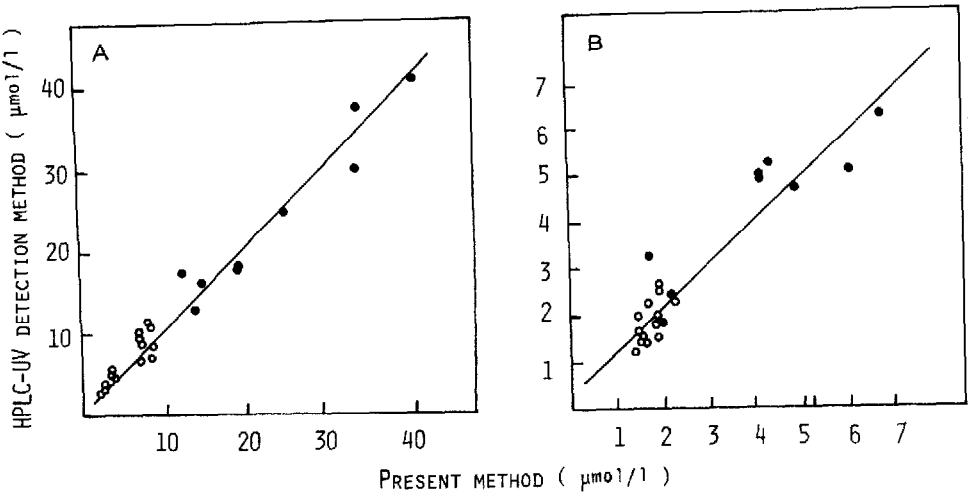

Fig. 6. Comparison of the results of Hyp (A) and Xan (B) in serum from normal (○) and gouty (●) subjects obtained by two methods.

TABLE I

DETERMINATION OF HYP AND XAN IN BIOLOGICAL FLUIDS FROM NORMAL SUBJECTS

Results are expressed as mean ± S.D. (n = 4).

Subject	Serum		Urine	
	Hyp (μmol/l)	Xan (μmol/l)	Hyp (μmol/l)	Xan (μmol/l)
1	4.04 ± 0.331	1.51 ± 0.151	58.70 ± 1.403	49.44 ± 2.051
2	8.60 ± 0.514	2.43 ± 0.092	144.52 ± 1.896	103.81 ± 22.056
3	3.60 ± 0.558	1.64 ± 0.237	190.51 ± 7.516	135.03 ± 3.550
4	7.71 ± 0.433	1.97 ± 0.145	85.15 ± 4.952	78.89 ± 4.464
5	8.16 ± 1.124	2.56 ± 0.316	101.76 ± 11.381	82.44 ± 1.775
Mean	6.42	2.02	116.13	89.92

We compared the results of Hyp and Xan in serum from normal subjects and gouty subjects obtained by the present method (x) with those using the HPLC—UV detection method (y) of Hartwick et al. [4]. Hyp and Xan in serum from gouty subjects have been known to exist at high levels compared with those in normal subjects; we found Hyp and Xan in serum from gouty subjects (n = 9) in the concentration ranges 12.93—41.14 μmol/l and 2.10—6.84 μmol/l, respectively. As shown in Fig. 6, the results obtained by these two methods were correlated closely: for Hyp $y = 0.99x + 0.08$ ($r = 0.99$), and for Xan $y = 0.93x + 0.05$ ($r = 0.93$).

The results for the determination of Hyp and Xan in biological fluids from normal subjects are presented in Table I. The values for Hyp (6.20 μmol/l) and Xan (2.04 μmol/l) in serum were slightly lower compared with those described by Hartwick et al. [4]: 7.16 μmol/l for Hyp and 2.62 μmol/l for Xan. The sensitivities of determination for Hyp and Xan were approximately

50 and 120 pg per injection, respectively. The assay of allopurinol, which reacts with xanthine oxidase to produce hydrogen peroxide, was also possible with a sensitivity of 130 pg per injection. Therefore, we expect that the present method is suitable for studying the influence of allopurinol on purine metabolism.

CONCLUSION

The present method was troublesome; however, it offered several advantages dependent on increasing the sensitivity and selectivity. The rapid assay was performed without using a gradient technique. The chromatographic columns were used for a large number of samples without showing any deterioration because it was possible to analyze small volumes (5 or 10 μl) of the diluted sample.

The mechanism and function of reducing the blank fluorescence by immobilized catalase will be discussed in the future.

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