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Note

Specific simultaneous assay of hypoxanthine and xanthine in serum by reversed-phase high-performance liquid chromatography using an immobilized xanthine oxidase reactor

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The simultaneous determination of hypoxanthine and xanthine in serum and urine has been required for various pharmacological and physiological reasons [1—3]. Several analytical procedures have been developed for these oxypurines at biological levels involving the use of high-performance liquid chromatography (HPLC) [4—7]. However, because many other compounds that absorb at 254 nm are present in serum and urine, good separations by HPLC usually require a long time and accurate quantitation is impossible. It is also difficult to determine these oxypurines simultaneously at 280 nm because of the absence of a strong UV absorption of hypoxanthine at this wavelength.

We first prepared xanthine oxidase bound to controlled pore-glass (CPG) and the properties of the immobilized enzyme have been investigated [8]. In order to simplify the simultaneous determination of hypoxanthine, xanthine and uric acid by HPLC and to provide a means by which they could be quantitated accurately, the performance of a reactor packed with immobilized xanthine oxidase has been also considered theoretically and experimentally [9]; when a post-column reactor packed with immobilized xanthine oxidase is to be employed, the complete conversion of both hypoxanthine and xanthine into uric acid, which can be detected simply and selectively, may be accomplished.

This paper describes a simultaneous assay of hypoxanthine and xanthine in serum from a patient with gout during alloprinol therapy, using an immobilized xanthine oxidase reactor coupled to HPLC.

EXPERIMENTAL

Chemicals

Hypoxanthine, xanthine and alloprinol were purchased from Nakarai Chemicals (Kyoto, Japan) and uric acid from Merck (Darmstadt, G.F.R.). Xanthine oxidase (cows' milk, 0.4 units/mg protein) was purchased from Boehringer-Mannheim-Yamanouchi (Tokyo, Japan), uricase (*Candida utilis*, 3.4 units/mg protein) from Toyobo (Tokyo, Japan) and catalase (bovine liver, 37,000 units/mg protein) from Sigma (St. Louis, MO, U.S.A.). Aminopropyl-CPG (80—120 mesh, 530 Å mean pore diameter) was obtained from Electro-Nucleonics (Fairfield, U.S.A.). All other reagents were of analytical-reagent grade.

Apparatus

The liquid chromatograph was a Model LC-3A (Shimadzu, Kyoto, Japan). Solutes were detected with a Shimadzu Model SPD-2A variable-wavelength UV detector equipped with a Model C-R1A integrator. The pre-column (1.0 × 0.4 cm I.D.) and the analytical column (20 × 0.4 cm I.D.) were prepared with Nucleosil 5 C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) by using a high-pressure slurry packing technique.

Immobilized enzyme reactor

The immobilization procedure was the same as that reported previously [8], in which the enzymes were attached to the aminopropyl-CPG by an intermolecular cross-linking method. The properties and the conversion efficiencies of the immobilized enzymes were determined by the flow-injection method [10]. The pH optima for the immobilized xanthine oxidase, uricase and catalase were 7.5, 8.0 and 8.0, respectively. Acetonitrile contents lower than 5% (v/v) did not degrade the activities of the immobilized enzymes. The immobilized xanthine oxidase and a mixture of the immobilized uricase and the immobilized catalase (mixing ratio 10:1) were packed into stainless-steel tubes (5.0 × 0.21 cm I.D.). Although the long-term stability of each immobilized enzyme was not studied in continuous operation, both enzyme reactors retained their performance without a decrease in activity for more than 7 weeks.

Serum purification

A 0.5-ml portion of serum was mixed with an equal volume of a 6% (v/v) solution of trichloroacetic acid, and the mixture was centrifuged for 15 min at 1500 *g*. Any 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, 10 μl of the aqueous solution were injected into the analytical column. Recovery experiments were carried out by adding known amounts of hypoxanthine and xanthine to the control serum. The recoveries of hypoxanthine and xanthine were 93 ± 9.9% (n = 4) and 96 ± 9.2% (n = 4), respectively.

RESULTS AND DISCUSSION

Fig. 1 demonstrates the change in capacity ratios (k') with the pH and the content of acetonitrile in the mobile phase for each solute. By considering the peak broadening by use of immobilized enzyme reactors, 0.01 *M* phosphate buffer (pH 5.5) containing 1% (v/v) acetonitrile was selected for this study.

Fig. 2 illustrates a chromatogram obtained for a serum extract from a patient with gout during alloprinol therapy, which was detected at 254 nm under the conditions described above. It shows that many other UV-absorbing

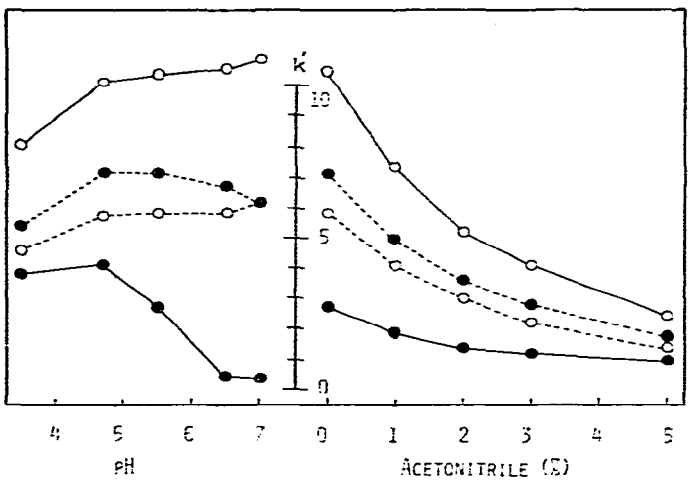

Fig. 1. Effects of pH and acetonitrile content on the capacity ratios (k') of hypoxanthine (- - - - o - - - -), xanthine (- - - - • - - - -), uric acid (—•—) and alloprinol (—o—). Column: Nucleosil 5 C_{18}, 20 × 0.4 cm I.D. Flow-rate: 0.7 ml/min. Detection: 254 nm.

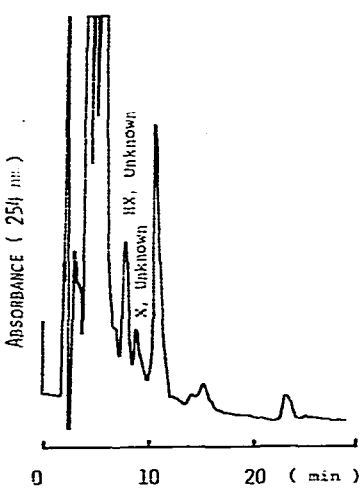

Fig. 2. Chromatogram of serum extract from patient with gout during alloprinol therapy. Injection volume: 10 µl. Column: Nucleosil 5 C_{18}, 20 × 0.4 cm I.D. Eluent: 0.01 *M* phosphate buffer (pH 5.5) containing 1% (v/v) acetonitrile. Flow-rate: 0.7 ml/min. HX = hypoxanthine; X = xanthine.

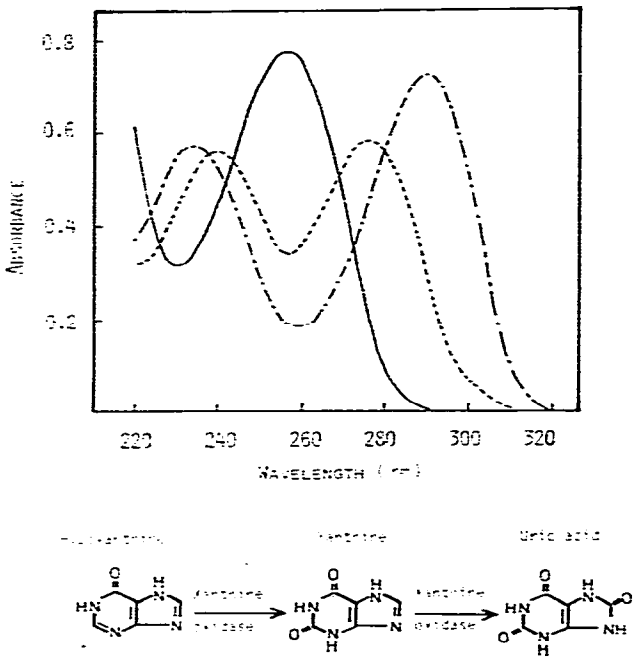

Fig. 3. Absorption spectra of hypoxanthine ($7.5 \cdot 10^{-5}$ *M*) (——), xanthine ($6.6 \cdot 10^{-5}$ *M*) (- - - - -) and uric acid ($6.0 \cdot 10^{-5}$ *M*) (— - —), and oxidative transformation of hypoxanthine and xanthine in uric acid by xanthine oxidase.

compounds interfere with the simultaneous quantitative determination of hypoxanthine and xanthine. On the other hand, when a reactor packed with immobilized xanthine oxidase was coupled to the analytical column, both hypoxanthine and xanthine were oxidized to uric acid and therefore could be detected at 290 nm simultaneously, as shown in Fig. 3. However, in this study it was impossible to determine such oxypurines accurately because of the high concentration of uric acid present in serum. Further, a large excess of uric acid degraded the original activity of the immobilized xanthine oxidase packed in the reactor. Therefore, another reactor packed with a mixture of immobilized uricase and immobilized catalase (mixing ratio 10:1) was utilized in order to remove the interferences of uric acid and its oxidation by-product

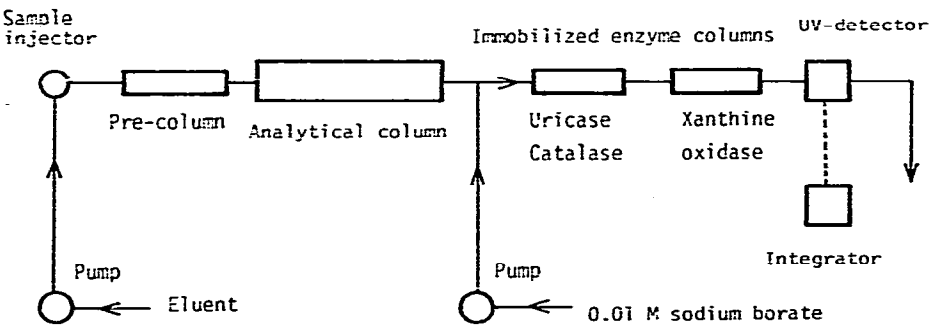

Fig. 4. Flow diagram of HPLC coupled with immobilized enzyme reactors.

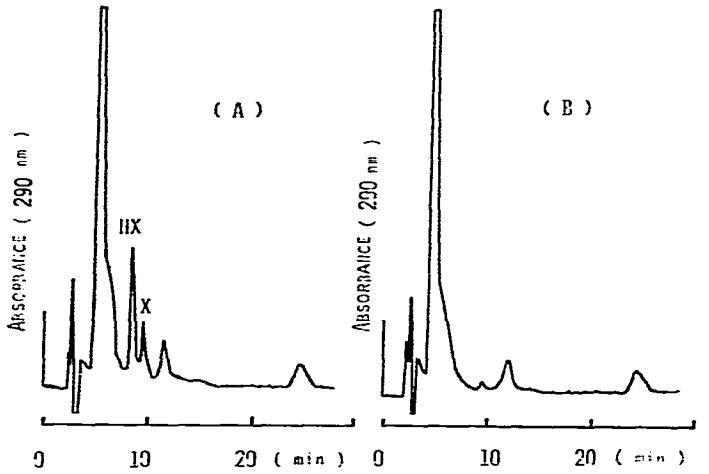

Fig. 5. Chromatograms of the same sample as in Fig. 2, which were obtained (A) with and (B) without the immobilized xanthine oxidase reactor in the system. Flow-rate of 0.01 *M* sodium borate: 0.27 ml/min. Other chromatographic conditions as in Fig. 2. HX = hypoxanthine; X = xanthine.

(hydrogen peroxide), which acts as an inhibitor of the immobilized xanthine oxidase packed in the reactor.

A diagram of the HPLC system coupled with two reactors used in this study is shown in Fig. 4. Fig. 5A illustrates a chromatogram obtained at 290 nm for the same serum sample under identical conditions, in which the effluent from the analytical column was adjusted to pH 7.7 with 0.01 *M* sodium borate and was passed continuously into the enzyme reactors. Fig. 5B shows a chromatogram obtained at the same wavelength, in which only the reactor packed with the immobilized xanthine oxidase had been removed. It shows that both peaks of hypoxanthine and xanthine, as shown in Fig. 5A, were eluted free from other UV-absorbing compounds.

The linearity of calibration graphs of concentration versus peak height was excellent for hypoxanthine, ranging from 0.20 to 1.60 μg/ml, and for xanthine, ranging from 0.19 to 1.52 μg/ml, and both regression coefficients were 0.998. The average coefficients of variation for the normalized peak height over this range of hypoxanthine and xanthine concentrations were

TABLE I

DETERMINATION OF HYPOXANTHINE AND XANTHINE IN SERA FROM PATIENTS WITH GOUT DURING ALLOPRINOL THERAPY

Sample	Hypoxanthine (μg/ml)*	Xanthine (μg/ml)*
1	3.27 ± 0.056	0.74 ± 0.033
2	1.06 ± 0.029	0.81 ± 0.029
3	1.57 ± 0.036	0.72 ± 0.018
4	1.69 ± 0.101	0.46 ± 0.013
5	2.34 ± 0.111	1.21 ± 0.040

*Means ± S.D. (n=4).

2.19 and 1.70%, respectively. The sensitivities of determination for hypoxanthine and xanthine were 68 and 76 ng/ml, respectively. Typical results for the determination of hypoxanthine and xanthine in sera of patients with gout during alloprinol therapy are presented in Table I.

In conclusion, a rapid and relatively simple HPLC method has been developed for the simultaneous identification and quantification of hypoxanthine and xanthine in biological fluids. The procedure can be easily instituted as a routine laboratory procedure in the diagnosis of purine metabolism.

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