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SIMULTANEOUS DETERMINATION OF HYPOXANTHINE, XANTHINE AND URIC ACID USING AN IMMOBILIZED XANTHINE OXIDASE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Immobilized xanthine oxidase was prepared by intermolecular cross-linking to porous aminopropyl-glass beads, and the properties were investigated in a batch process. Simultaneous analysis of hypoxanthine, xanthine and uric acid was achieved by using small column packed with the immobilized xanthine oxidase as a reactor for high-performance liquid chromatography (HPLC).

There is evidence that the plasma levels of oxypurines (hypoxanthine and xanthine) and uric acid may provide a sensitive indicator of certain pathologic states, e.g., xanthinuria, renal failure and toxaemia of pregnancy.[1] Especially with uric acid, the determination of hypoxanthine/xanthine in plasma may be of value in diagnoses of haemorrhagic shock and intrauterine hypoxia of newborn infants.[2] Therefore a simple and accurate method has been demanded. HPLC has been widely used in the determination of the plasma concentrations of these oxypurines, but the several difficulties have been found in satisfactory separation or sensitivity by direct analysis.[3] When an immobilized xanthine oxidase packed in column is used in combination with usual HPLC column, simultaneous detection of these oxypurines and uric acid would be anticipated with high sensitivity, due to its highly specific conversions of hypoxanthine and xanthine to uric acid, but this reactor has never been utilized.

We have first prepared xanthine oxidase bound to the controlled-pore-glass (CPG), and have developed a method of simultaneous determination of hypoxanthine, xanthine and uric acid by HPLC utilizing it as a post-column reactor.

Immobilized xanthine oxidase was prepared by intermolecular cross-linking to the CPG derivatives: a 500 mg portion of aminopropyl-CPG (80-120 mesh, 530 Å mean pore diameter) was suspended in 1 ml of 5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. The solution was allowed to stand for 1 hr under vacuum at 25° with occasional gently mixing. The derivative glass was washed with cold distilled water. A 2 mg of xanthine oxidase (EC 1.2.3.2, cow milk, about 0.4 U/mg) dialyzed overnight against 500 ml of 0.1 M phosphate buffer, pH 7.2, containing 10 mM EDTA was added to the washed glass in 2 ml of 0.1 M phosphate buffer, pH 7.0. The mixture was allowed to react at 4° overnight. The glass was washed with 100 ml of cold distilled water to remove any extraneous enzyme and followed by a wash of 100 ml of 1 M sodium chloride, and finally 1000 ml of cold distilled water.

The pH dependency of activity was studied at 25° at xanthine concentration of

5.4×10^{-5} M. All assays were performed in 50 mM phosphate buffer containing 10 mM EDTA by the same instruments and method as described previously.[4] As shown in Fig. 1, there is no significant difference between the pH-activity curves of the immobilized xanthine oxidase and that of the native enzyme, and both activity maxima were at pH 7.4.[5] The effect of temperature on the stability of enzyme free in solution and immobilized one was studied (Fig. 2). A 0.2 ml of suspended one in 3.2 M ammonium sulfate containing 10 mM EDTA was incubated at different temperature for period of 30 min. After cooled rapidly, 0.1 ml aliquots was pipetted into a mixture of 3.3 ml of 50 mM phosphate buffer, pH 7.4, containing 10 mM EDTA and 7.0×10^{-5} M xanthine at 25°, and the residual activity was determined. After each assay the preparations were kept at 4° in 3.2 M ammonium sulfate containing 10 mM EDTA. The retention activity was 70 % or above after repetitive use for more than 30 days. The apparent Michaelis constant, K_m', was analyzed for different batches of enzymes and the least-squares' fitting to an integrated form of the Michaelis-Menten equation afforded about 2.5×10^{-5} M for xanthine at pH 7.4 (25°).

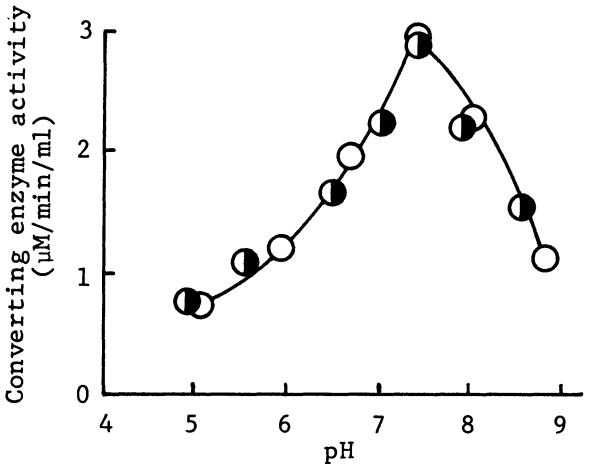

Fig. 1. Effect of pH on the oxidation of xanthine by xanthine oxidase free in solution(o) and immobilized to glass(●).

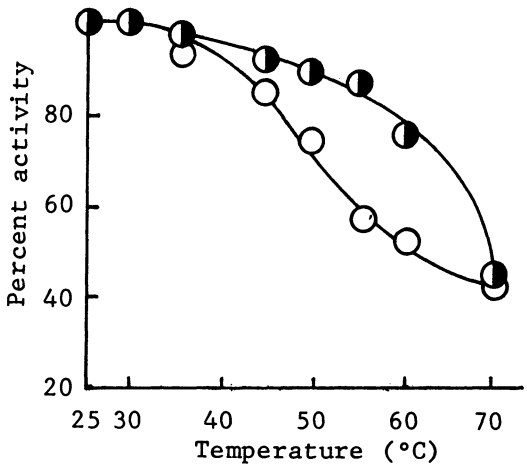

Fig. 2. Temperature sensitivity of soluble(o) and immobilized xanthine oxidase(●).

The packed-bed reactor was made from Teflon tubing (4.2 cm x 1 mm I.D.) and filled with immobilized xanthine oxidase beads, and connected to a column through a mixing tee. The chromatographic set-up is shown in Fig. 3. A Shimadzu LC-3A liquid chromatograph was used together with a Shimadzu UVD-2 variable-wavelength UV-detector and a Shimadzu C-R1A integrator. The column was prepared with C_{18} Nucleosil (5 μm, 20 cm x 4 mm I.D.). The eluent, 0.01 M potassium phosphate (pH 4.8), was pumped through the column connected to the tee at a flow rate of 0.7 ml/min and the effluent through the column was adjusted to pH 7.4 with 0.01 M sodium borate (pH 9.2) pumped into the tee at flow rate 0.23 ml/min. The results are shown in Fig. 4. Chromatograms A, B and C were obtained at 254, 280 and 290 nm when the immobilized enzyme reactor had been removed, respectively. Chromatogram D was obtained at 290 nm when the flow had passed the enzyme reactor under the identical conditions. The additional band broadening resulted by the enzyme reactor was 5.2 %. The average overall conversion yields for hypoxanthine and

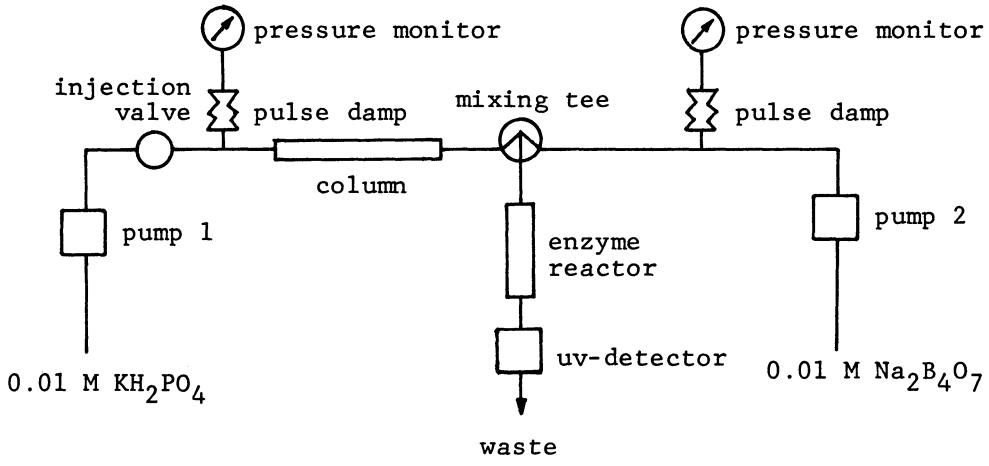

Fig. 3. Schematic diagram of the chromatographic system.

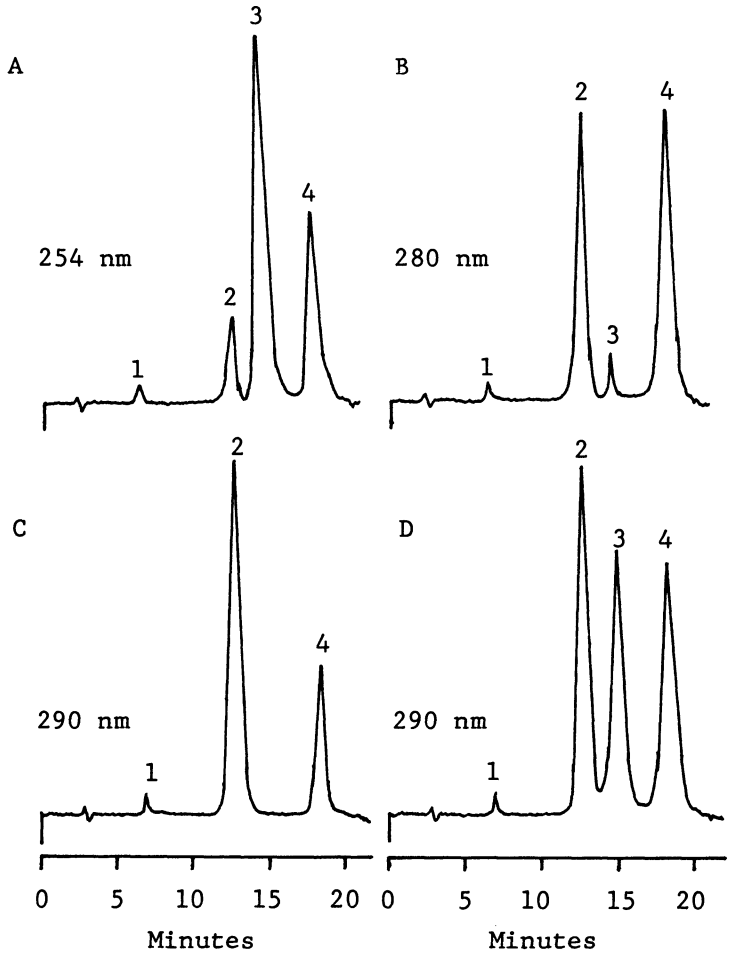

Fig. 4. HPLC chromatograms of a mixture of hypoxanthine, xanthine and uric acid.

1: unknown; 2: uric acid(2.47 μg/ml); 3: hypoxanthine (2.024 μg/ml); 4: xanthine(2.250 μg/ml).

xanthine to uric acid were over 89 and 100 % at a flow rate of 0.93 ml/min, respectively. A series of samples containing hypoxanthine, xanthine and uric acid were achieved and the results obtained are summarized in Table 1. The calibration curves of peak area vs. amount injected for these oxypurines were linear up to 3 μg/ml. The coefficients of variation were 13.4 and 9.0 % for hypoxanthine of 0.405 and xanthine of 0.450 μg/ml, respectively.

In the near future this work will be applied to blood or urine from patients with gout and/or renal failure during alloprinol therapy.

Table 1. Determination of mixtures of hypoxanthine, xanthine and uric acid

Compound	Amount added (μg/ml)	Amount found[a] (μg/ml)	Recovery (%)
Hypoxanthine	0.405	0.397 ± 0.053	98.0
Xanthine	0.450	0.498 ± 0.045	110.7
Uric acid	0.495	0.493 ± 0.155	99.6
	0.648	0.543 ± 0.063	83.8
	0.720	0.745 ± 0.050	103.5
	0.792	0.785 ± 0.029	99.1
	0.810	0.717 ± 0.048	88.5
	0.900	0.921 ± 0.059	102.3
	0.990	0.991 ± 0.136	100.1
	1.214	1.048 ± 0.018	89.3
	1.350	1.334 ± 0.019	98.8
	1.485	1.498 ± 0.118	100.9
	1.620	1.420 ± 0.041	87.7
	1.799	1.760 ± 0.006	97.8
	1.980	1.983 ± 0.144	100.2

a) Mean ± S.D. (n = 4)

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