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Determination of purine nucleosides and their bases by high-performance liquid chromatography using co-immobilized enzyme reactors

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SUMMARY

A selective and sensitive assay of inosine, guanosine, hypoxanthine, guanine and xanthine by high-performance liquid chromatography with immobilized enzyme reactors was developed. The separation was achieved on a Capcell Pak C_{18} column (15 cm × 0.46 cm I.D.) with a mobile phase of 0.1 *M* phosphate buffer (pH 8.0) containing 7 m*M* sodium 1-hexanesulphonate and 0.1 m*M* *p*-hydroxyphenylacetic acid. The fluorimetric detection of hydrogen peroxide using immobilized peroxidase and *p*-hydroxyphenylacetic acid was applied to the assay of these compounds, which were oxidized to yield hydrogen peroxide in the presence of immobilized enzyme (purine nucleoside phosphorylase, guanase and xanthine oxidase). Enzyme reactions occurred sufficiently without post-column addition of reagents. Enzymes that catalysed the conversion of purine compounds were co-immobilized on aminopropyl controlled-pore glass packed in stainless-steel tubing. The detection limits were 30–200 pg per injection.

INTRODUCTION

A simultaneous determination of guanosine (Guo), inosine (Ino), guanine (Gua), hypoxanthine (Hyp) and xanthine (Xan) is necessary in purine metabolism disorders, such as xanthine oxidase (XOD) deficiency [1], hypoxanthine–guanine phosphoribosyltransferase deficiency [2] and purine nucleoside phosphorylase (PNP) deficiency [3]. The determination of these compounds in biological fluids requires sensitive and specific methods because of their low concentration and the presence of many compounds.

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Recently, high-performance liquid chromatography (HPLC) has become popular for the assay of these compounds [4–8]. However, most of the HPLC methods use UV detection, which is often unsatisfactory for sensitive and selective assays. Post-column derivatization techniques with immobilized enzymes have often been used to improve the sensitivity and selectivity of detection in HPLC [9–11].

We have already reported a post-column reaction system for the assay of Hyp, Xan or Ino [12,13]. After separation by HPLC, hydrogen peroxide produced from Hyp, Xan and Ino by immobilized PNP and XOD was determined fluorimetrically using immobilized peroxidase (POD) and *p*-hydroxyphenylacetic acid. In this system, two pumps were used to add reagents for enzyme reactions. The disadvantage of this method was the additional broadening of chromatographic peaks caused by post-column addition of reagents for enzyme reactions. Also, the complete mixing of the two flows was troublesome.

This paper describes a sensitive and selective procedure for the simultaneous determination of Guo, Ino, Gua, Hyp and Xan by HPLC coupled with the enzyme reactors without the addition of reagents. The enzyme reactions in the post-column reactors are as follows:

$$\text{Guo} + \text{phosphate} \xrightleftharpoons{\text{PNP}} \text{Gua} + \text{ribose-1-phosphate} \quad (1)$$

$$\text{Ino} + \text{phosphate} \xrightleftharpoons{\text{PNP}} \text{Hyp} + \text{ribose-1-phosphate} \quad (2)$$

$$\text{Gua} + \text{H}_2\text{O} \xrightarrow{\text{guanase}} \text{Xan} + \text{NH}_3 \quad (3)$$

$$\text{Hyp} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{XOD}} \text{Xan} + \text{hydrogen peroxide} \quad (4)$$

$$\text{Xan} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{XOD}} \text{uric acid} + \text{hydrogen peroxide} \quad (5)$$

$$\text{Hydrogen peroxide} + p\text{-hydroxyphenylacetic acid} \xrightarrow{\text{POD}} \text{fluorescent compound [14]} \quad (6)$$

In this study, all experiments were carried out with phosphate buffer in order to prevent the reverse reactions of PNP (eqns. 1 and 2).

EXPERIMENTAL

Materials

Standard solutions of Guo, Hyp, Gua and Xan (Nakarai Chemicals, Japan) were prepared by dissolving the compounds in minimum amounts of weak sodium hydroxide solution and then diluting the solutions with 0.1 *M* phosphate buffer (pH 8.0). A standard solution of Ino (Nakarai Chemicals) was prepared

by dissolving it in distilled water. *p*-Hydroxyphenylacetic acid (Nakarai Chemicals) was purified by sublimation and dissolved in mobile phase before use. XOD (cow's milk, 0.4 U/mg of protein), PNP (calf spleen, 20 U/mg of protein) and guanase (GN, rabbit liver, 0.15 U/mg of protein), all from Boehringer-Mannheim Yamanouchi (Japan), were dialysed overnight against 0.1 *M* phosphate buffer (pH 7.0) and immobilized on aminopropyl controlled-pore glass (80–530 Å mean pore diameter, Electro-Nucleonics, U.S.A.). POD (horseradish, 264 U/mg of protein, Toyobo, Japan) was immobilized on aminopropyl controlled-pore glass through the aldehyde groups produced from the carbohydrate residues by periodate oxidation [15]. Unless otherwise stated all reagents were of analytical-grade quality.

High-performance liquid chromatography

The liquid chromatograph used was a Model LC-6A (Shimadzu, Japan) equipped with a fluorescence spectrophotometer Model RF-530 (Shimadzu) or an UV spectrophotometer Model SPD-6A (Shimadzu). A guard column (1 cm × 0.4 cm I.D.) and an analytical column (15 cm × 0.46 cm I.D.) were packed with Capcell Pak C_{18} (5 μm, Shiseido, Japan), which is a silica-based packing and can be used in alkaline mobile phases (pH < 10), using a high-pressure slurry-packing technique.

The liquid chromatographic system for the determination of the immobilized enzyme activity consisted of a pump (Model LC-3A) and a UV absorbance detector set at 254 nm (Model SPD-2A) from Shimazdu, a separation column (10 cm × 0.4 cm I.D., Nucleosil $5C_{18}$, Macherey, Nagel, F.R.G.) and the immobilized enzyme reactor (Fig. 1). The separation between the substrate and product of each enzyme reaction was satisfactory by eluting 0.1 *M* phosphate buffer (pH 5–9).

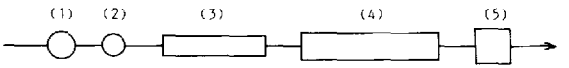

Fig. 1. System for the determination of the immobilized enzyme activity. 1 = Pump; 2 = injector; 3 = enzyme reactor; 4 = analytical column; 5 = UV detector.

Preparation of enzyme reactor

XOD, PNP and GN were co-immobilized on aminopropyl controlled-pore glass beads by cross-linking with glutaraldehyde. Beads were packed into the stainless-steel column (10 cm × 0.21 cm I.D.) by the dry-packing method used in preparation of the HPLC column. A 5% solution of glutaraldehyde in 0.1 *M* phosphate buffer was run through the glass beads column with a peristaltic pump at a flow-rate of 0.5 ml/min for 2 h, and then 0.1 *M* phosphate buffer (pH 7.0) was run at a flow-rate of 0.5 ml/min for 30 min in order to remove excess glutaraldehyde. A mixture of dialysed enzymes containing 2 U each of XOD, PNP and GN was circulated through the glass beads column with the

peristaltic pump (flow-rate of 0.2 ml/min) for 12 h at 4°C. The column was then flushed with water (100 ml), 1 *M* sodium chloride (50 ml) and 0.1 *M* phosphate buffer (pH 7.0, 50 ml). To determine the activities, XO, PNP and GN were immobilized individually as described previously [16] and then packed into the PTFE tubing. The immobilized POD was prepared as reported previously [12] and packed into the stainless-steel (10 cm×0.21 cm I.D.) or PTFE tubing. The enzyme reactors were stored in 0.1 *M* phosphate buffer (pH 7.0) at 4°C.

RESULTS AND DISCUSSION

Characterization of immobilized enzyme reactor

The effect of pH on the activity of immobilized enzyme was examined in the pH range 5–9 using the system illustrated in Fig. 1. All immobilized enzymes used in this study exhibited the highest activity at pH 7–8 (Fig. 2). Conversely, the fluorescence of the fluorophore increased with pH and was maximal at pH 9.5. The effect of the concentration of *p*-hydroxyphenylacetic acid on the POD reaction was studied in the range 10^{-6}–10^{-2} *M*. As the concentration was increased, the fluorescence increased slowly and so did the background fluorescence in the mobile phase. Thus, 0.1 m*M* was chosen for the concentration of *p*-hydroxyphenylacetic acid. *p*-Hydroxyphenylacetic acid did not influence other enzyme reactions. Although the activities of immobilized enzymes increased slowly with increasing temperature (20–40°C), 25°C was selected in order to prolong the lifetime of the immobilized enzymes.

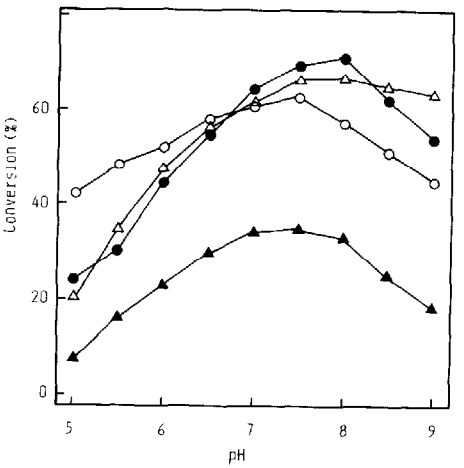

Fig 2 Effect of pH on the enzyme activity. Reactor, XOD (1 cm×0.1 cm, ○), PNP (1.3 cm×0.1 cm, ▲), GN (2.5 cm×0.1 cm, △), POD (3 cm×0.1 cm, ●). Flow-rate, 0.2 ml/min.

Separation

The separation of purine nucleosides and their bases was investigated by eluting the solution in which the immobilized enzyme reactions occurred sufficiently. Fig. 3 shows the effect of pH of the mobile phase on the capacity factor (k'). The separation of Hyp, Gua and Xan was poor at pH below 7.5. In addition, uric acid, which is present in biological fluids at high concentrations, interfered with the determination of Xan at pH above 8.2. Also, sodium 1-hexanesulphonate (SHS) was added to the phosphate buffer (pH 8.0) in order to separate the peaks of Gua and Hyp (Fig. 4). The k' value of Hyp decreased with increasing concentration of SHS and that of Gua was influenced only slightly. In addition, modification of the mobile phase by addition

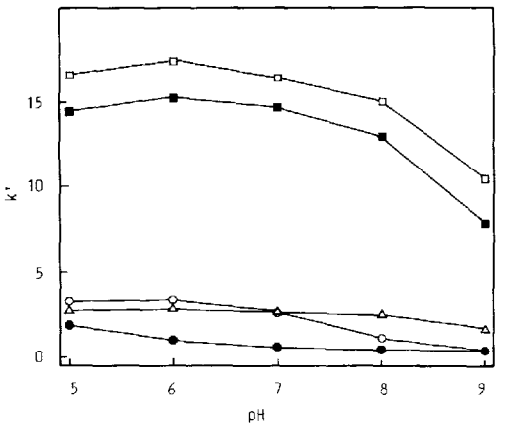

Fig. 3. Effect of pH of mobile phase on k' values of Guo (□), Ino (■), Gua+Hyp (△), Xan (○) and uric acid (●).

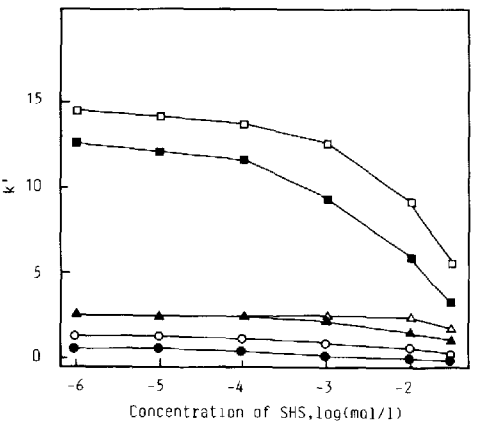

Fig. 4. Effect of concentration of SHS on k' values of Guo (□), Ino (■), Gua (△), Hyp (▲), Xan (○) and uric acid (●).

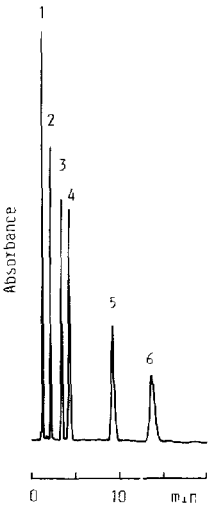

Fig. 5. Separation of (1) uric acid, (2) Xan, (3) Hyp, (4) Gua, (5) Ino and (6) Guo. Column, Capcell Pak C_{18} (15 cm × 0 46 cm I.D.); mobile phase, 0.1 *M* phosphate buffer (pH 8.0) containing 7 m*M* SHS, flow-rate, 1.0 ml/min; injection volume, 10 μl of 50 μ*M* of each compound; detection wavelength, 254 nm

of SHS resulted in a decrease in the analysis time. Therefore, the best mobile phase with respect to both enzyme reactions and analytical sensitivity was 0.1 *M* phosphate buffer (pH 8.0) containing 7 m*M* SHS and 0.1 m*M* *p*-hydroxyphenylacetic acid (Fig. 5). The enzyme reactions of XOD, PNP and GN were not influenced by the presence of SHS at concentrations below 10 m*M* in the mobile phase. However, the activity of POD was decreased only slightly by increasing the concentration of SHS, and this effect was reversible. Also, the separation was not influenced by the presence of *p*-hydroxyphenylacetic acid at concentrations below 5 m*M* in the mobile phase.

HPLC coupled with the immobilized enzyme reactor

A diagram of the HPLC system coupled with enzyme reactors is shown in Fig. 6. The mobile phase used was 0.1 *M* phosphate buffer (pH 8.0) containing 7 m*M* SHS and 0.1 m*M* *p*-hydroxyphenylacetic acid (flow-rate of 1.0 ml/min). After separation on the analytical column, the effluent was run through the co-immobilized enzyme reactor and the POD reactor without any modifications. Purine nucleosides and their bases were converted into hydrogen peroxide by GN, PNP and XOD (eqns. 1–5). Then, *p*-hydroxyphenylacetic acid was oxidized by hydrogen peroxide in the POD reactor to form a highly fluorescent compound (excitation maximum 320 nm, emission maximum 407 nm) (eqn. 6). Fig. 7A shows a chromatogram of the standard mixture containing Guo, Ino, Gua, Hyp, Xan and uric acid obtained under the conditions described. Additional peak broadening was observed (compared with the peaks

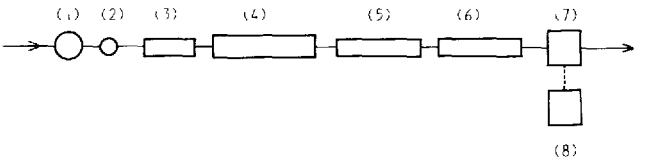

Fig. 6. Flow diagram of HPLC coupled with the immobilized enzyme reactors. 1 = Pump; 2 = injector; 3 = guard column, 4 = analytical column; 5 = co-immobilized reactor (PNP, GN and XOD); 6 = POD reactor; 7 = fluorescence detector; 8 = integrator.

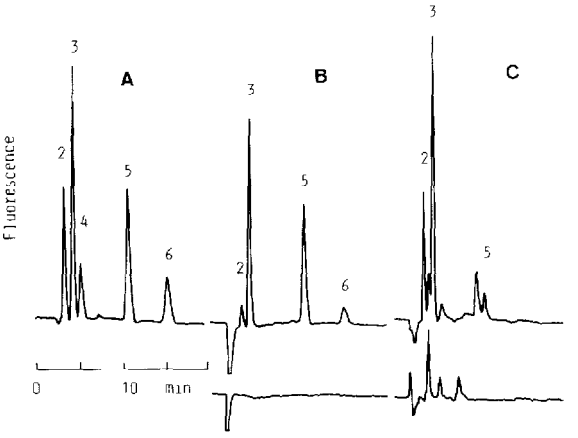

Fig 7. Chromatograms of (A) standard solution (1 μM of each compound), (B) serum extract and (C) urine, obtained by the present method (upper) and obtained when the co-immobilized reactor was removed from the system (lower). Mobile phase 0.1 M phosphate buffer (pH 8.0) containing 7 mM SHS and 0.1 mM p-hyddroxyphenylacetic acid; fluorescence detection wavelengths, 320 nm (excitation) and 407 nm (emission). Other conditions as in Fig. 5.

illustrated in Fig. 5) and was an irreducible minimum in the present system. The co-existence of enzymes is favourable to multi-enzyme systems (eqns. 1–5). Therefore, all the enzymes except POD were co-immobilized on aminopropyl controlled-pore glass, which was packed by the normal dry-packing method. A smaller reactor, which is of importance for reducing the broadening with the high activities, was prepared by this procedure. Unfortunately, co-immobilization including POD was impossible because of the incompatibility of im-

mobilization procedures. Also, the broadening was influenced by the flow-rate of the mobile phase. A lower flow-rate in the immobilized enzyme reactor was favourable to the enzyme reactions, but the peak broadening was increased by decreasing the flow-rate. In addition, the flow-rate influences both the HPLC separation and the analysis time. The optimal flow-rate of the present system was 1 ml/min.

The chromatograms of a serum extract from a normal subject are shown in Fig. 7B. The upper chromatogram was obtained using the system illustrated in Fig. 6, and the lower chromatogram was obtained when the co-immobilized enzyme reactor was removed. No peak appeared after elution of Guo. These results show that the selective assay of Guo, Ino, Gua, Hyp and Xan in serum extracts can be performed within 20 min and that there are no interfering peaks in the areas where Guo, Ino, Gua, Hyp and Xan elute. A dip in the baseline at 2 min was due to the quenching of the background fluorescence by uric acid. Fig. 7C shows the chromatograms obtained from urine extracts. The determination of Gua in urine was impossible because of its co-elution with an unknown compound. Biological samples were prepared as described previously [12].

Evaluation of analytical system

The calibration curves of each compound were linear over the concentration range 0.2–10 μM, with intercepts not significantly different from zero (the correlation coefficients were greater than 0.998). The limits of determination for Guo, Ino, Gua, Hyp and Xan were ca. 200, 50, 120, 30 and 50 pg per injection, respectively.

The reproducibilities of retention times and peak areas were investigated (Table I). In general, reversed-phase silica columns should not be used with aqueous alkaline mobile phases (pH > 7.5) for a long period because of disso-

TABLE I

REPRODUCIBILITIES OF RETENTION TIMES AND PEAK AREAS

The intra-assay and inter-assay C.V. were estimated by assaying a standard solution (10 μl) containing 1 μM of each compound on the same day ($n = 14$) and on different days ($n = 12$), respectively.

Substance	C.V. of retention times (%)		C.V. of peak area (%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Xan	1.5	2.4	3.8	7.3
Hyp	1.2	1.8	1.8	5.1
Gua	1.1	1.2	6.1	11.4
Ino	0.4	0.5	2.4	8.1
Guo	0.8	1.1	4.8	9.8

lution of the packing. The analytical column packed with Capcell Pak C_{18} was stable in aqueous mobile phase (pH 8.0) and provided reproducible retention times with inter-assay coefficients of variation (C.V.) of less than 3%. The intra-assay C.V. of peak areas were acceptable, but the inter-assay C.V. of peak areas were high. Therefore, the concentrations of each compound should be determined using calibration curves prepared daily. The reproducibility of the peak area of Xan in biological samples containing high levels of uric acid may be rather large.

In this study, the analytical column was used for at least 500 determinations without any sign of irreversible deterioration. The guard column was renewed every 50–70 determinations. The co-immobilized reactor and the immobilized POD reactor were used for ca. 130 and 80 determinations, respectively, without any significant decrease in the activity.

In conclusion, the present method is highly sensitive and selective, and would be convenient for biological investigations.

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