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Short communication

Determination of human plasma xanthine oxidase activity by high-performance liquid chromatography

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Abstract

An assay for human plasma xanthine oxidase activity was developed with pterin as the substrate and the separation of product (isoxanthopterin) by high-performance liquid chromatography with a fluorescence detector. The reaction mixture consists of 60 μl of plasma and 240 μl of 0.2 *M* Tris–HCl buffer (pH 9.0) containing 113 μM pterin. With this assay, the activity of plasma xanthine oxidase could be easily determined despite its low activity. As a result, it could be demonstrated that the intravenous administration of heparin or the oral administration of ethanol did not increase plasma xanthine oxidase activity in normal subjects, and also that plasma xanthine oxidase activity was higher in patients with hepatitis C virus infection than in healthy subjects or patients with gout. In addition, a single patient with von Gierke's disease showed a marked increase in the plasma activity of this enzyme, relative to that apparent in normal subjects.

Keywords: Xanthine oxidase; Enzymes

1. Introduction

Xanthine oxidase (EC 1.2.3.2) catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid, producing the oxidants O_2^- and H_2O_2. The generation of these oxygen species has been implicated in the pathogenesis of tissue injury associated with many diseases, including that associated with reperfusion of ischemic tissues [1]. Xanthine oxidase is present in all rat tissues examined, whereas in humans it occurs predominantly in the liver and the small intestine, although antibodies to the enzyme have also demonstrated its presence in the endothelium, bronchial wall, kidney and heart [2]. The activity of xanthine oxidase in plasma is increased in individuals with viral hepatitis or toxic liver injury, indicating that plasma enzyme activity is a sensitive marker of acute hepatocellular damage [3–6]. In addition, plasma xanthine oxidase activity may play a role in the oxidant-mediated tissue damage [7]. However, measurement of plasma xanthine oxidase activity is not included in routine laboratory analyses because it must be determined in humans by a radiochemical method that is time consuming and labor intensive. We have now developed a method for the measurement of plasma xanthine oxidase activity by high-performance liquid chromatography (HPLC) with a fluorescence detector since plasma xanthine oxidase activity is too low for measurement by HPLC with a spectrophotometric detector [8].

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With this method, we compared plasma xanthine oxidase activity in individuals with various diseases.

2. Experimental

2.1. Subjects

The 42 subjects (male 30, female 12) comprised 16 control individuals, 15 patients with gout before treatment, 10 patients with hepatitis C virus infection, and 1 patient with von Gierke's disease. The control subjects had normal laboratory data. The patients with gout were diagnosed by the criteria of the American Rheumatism Association, and the patient with von Gierke's disease by enzymatic assay of platelet glucose 6-phosphatase [9]. The patients with hepatitis C virus infection were diagnosed by HCV RIVA test II (Chiron, CA, USA) and laboratory data, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum; the latter were from 45 and 46 to 180 and 200 Karmen U, respectively.

2.2. Chemicals

Pterin, isoxanthopterin, Tris, and uricase were obtained from Sigma (St. Louis, MO, USA). Allopurinol was prepared by Tanabe Pharmaceuticals (Tokyo, Japan). Other chemicals were from Wako (Osaka, Japan). Because pterin supplied by Sigma was not homogeneous, it was further purified by HPLC as described below for the measurement of isoxanthopterin; two HPLC separations were performed, the first with 20 m*M* potassium phosphate buffer (pH 2.2) and the second with water. The purified pterin was then lyophilized.

2.3. Statistical analysis

Values are expressed as means±S.D. The significance of differences in plasma xanthine oxidase activity among the different groups of subjects was assessed by the Mann–Whitney *U* test.

A *P* value of <0.05 was considered statistically significant.

2.4. Apparatus and technique

The HPLC system consisted of an LC-6A HPLC apparatus (Shimadzu, Kyoto, Japan), a fluorescence HPLC monitor (RF 530, Shimadzu), and a C-R3A Chromatopac recorder (Shimadzu). The mobile phase was 20 m*M* potassium phosphate buffer (pH 2.2), the flow-rate was 1.0 ml/min and the excitation and emission wavelengths were 345 and 410 nm, respectively. A Wakosil 5C18-200 column (200×4.6 mm I.D.) (Wako) was used at room temperature. A linear calibration curve for isoxanthopterin was obtained between 1 n*M* and 250 n*M*.

2.5. Sample preparation and enzyme reaction

Blood was collected from the subjects into 10-ml test tubes containing EDTA and then immediately centrifuged for 10 min at 1700 *g* and 4°C for isolation of plasma. After a 5 min preincubation at 37°C of 240 μl 0.2 *M* Tris–HCl buffer (pH 9.0) containing 113 μ*M* pterin, the enzyme reaction was initiated by the addition of 60 μl of plasma. After incubation for 240 min, 100 μl of the reaction mixture were removed and added to 1.5-ml tubes containing 100 μl of 4% $HClO_4$. The resulting mixture was vigorously shaken with an agitator and then centrifuged at 15 000 *g* (Model RM150, Tomy Seiki, Tokyo, Japan). A portion (150 μl) of the supernatant was neutralized with 6 μl of 5 *M* K_2CO_3, and 20 μl of the neutralized supernatant were injected. Plasma xanthine oxidase activity was measured in duplicate and expressed as picomoles of isoxanthopterin produced per hour per milliliter-(pmol/h/ml). Plasma samples were also collected before and 15 min after intravenous injection of heparin (30 U per kilogram of body mass) from 5 normal subjects who had fasted for more than 12 h, as well as before and 30 and 90 min after oral administration of ethanol (0.8 g/kg) in distilled water from another five normal subjects who had fasted for more than 10 h. These samples were also assayed for xanthine oxidase activity.

3. Results

3.1. Optimization of conditions for the measurement of plasma xanthine oxidase activity

Preliminary studies were performed to optimize the conditions for accurate measurement of plasma xanthine oxidase activity. Three plasma samples (120 μl) containing 1.1–2.0 μ*M* hypoxanthine, 0.4–0.8 μ*M* xanthine and 250–350 μ*M* uric acid were incubated with 480 μl of 0.2 *M* Tris–HCl buffer (pH 9.0) containing 113 μ*M* pterin for up to 5 h, during which time the concentration of isoxanthopterin in the reaction mixtures increased in a linear manner (Fig. 1). Xanthine oxidase activity was higher in a reaction mixture consisting of 60 μl plasma and 240 μl 0.2 *M* Tris–HCl buffer (pH 9.0) containing pterin than in corresponding reaction mixtures containing 0.2 *M* Tris–HCl buffer at pH 6.0, 7.0, 8.0 or 10.0 (Fig. 2), indicating that the optimal pH of the Tris–HCl buffer is 9.0 or close to 9.0. To measure the total activity of xanthine oxidase plus xanthine dehydrogenase in plasma, we added NAD (final assay concentration, 1.3 m*M*) to the 0.2 *M* Tris–HCl buffer (pH 9.0) containing pterin before the reaction was initiated. The total activity did not differ from the activity of xanthine oxidase alone (data not shown), indicating that dehydrogenase activity in plasma is negligible. Xanthine oxidase activity increased as the final concentration of pterin in the reaction mixture increased up to 80 μ*M*; further increasing the substrate concentration, did not further increase enzyme activity (data not shown). On the basis of these results, a reaction mixture consisting of 60 μl plasma and 240 μl 0.2 *M* Tris–HCl buffer (pH 9.0) containing 113 μ*M* pterin was incubated for 4 h for subsequent determinations of plasma xanthine oxidase activity.

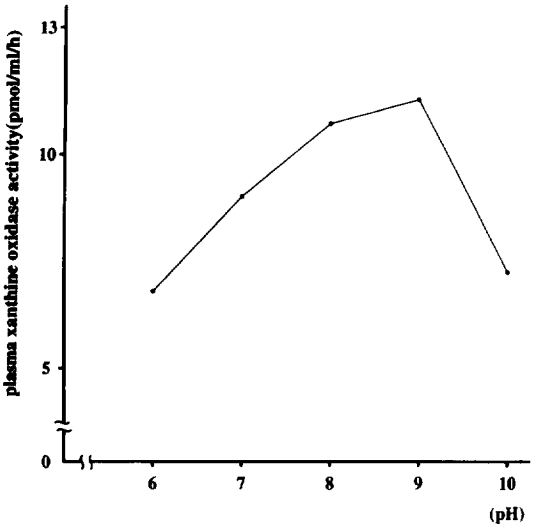

Fig. 2. Effect of pH on plasma xanthine oxidase activity. Three different plasma samples (60 μl) were incubated for 4 h with 240 μl of 0.2 *M* Tris–HCl buffer (pH 6.0, 7.0, 8.0, 9.0 or 10.0) containing 113 μ*M* pterin. Values are means.

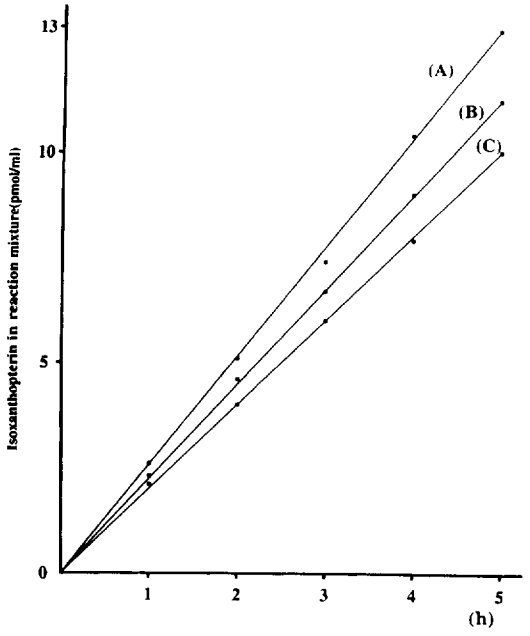

Fig. 1. Time course of the increase in isoxanthopterin concentration in the reaction mixture. Three different plasma samples (120 μl) were incubated at 37°C with 480 μl of 0.2 *M* Tris–HCl buffer (pH 9.0) containing 113 μ*M* pterin. After 1, 2, 3, 4 and 5 h, 100 μl were removed and assayed for isoxanthopterin. (A) First sample; (B) second sample; (C) third sample.

3.2. Effects of purine bases (uric acid, hypoxanthine, and xanthine) on the oxidation of pterin to isoxanthopterin

To investigate whether uric acid inhibits xanthine oxidase activity, we added plasma to buffer con-

taining pterin and uric acid (final assay concentration, 180 μM) and measured enzyme activity. In addition, 60 μl plasma containing 0.074 U of added uricase was incubated for 15 min at 37°C, to metabolize uric acid to allantoin before the addition to buffer containing pterin and the assay of plasma xanthine oxidase activity was performed. Neither the addition of uric acid nor uricase had significant effect on plasma xanthine oxidase activity (data not shown), suggesting that inhibition of xanthine oxidase by uric acid was negligible. Because both hypoxanthine and xanthine might competitively inhibit the oxidation of pterin to isoxanthopterin by xanthine oxidase, the effects of these components on plasma xanthine oxidase activity were examined. Plasma xanthine oxidase activity was 99.6, 99.7, and 83.2% of control in the presence of 0.7, 1.4, and 3.6 μM hypoxanthine in the reaction mixture, and 100.1% of control in the presence of 0.7 μM xanthine. These values were calculated from means of xanthine oxidase activities in 3 different normal plasma samples.

3.3. Linearity of detection, recovery of isoxanthopterin and HPLC-drawn chromatogram

A calibration curve prepared with various concentrations of isoxanthopterin in aqueous solution was linear up to 0.25 μM. The lower detection limit for isoxanthopterin was 0.01 pmol and the relative standard deviation was below 1.7% at all concentrations between 1 and 250 nM. The regression line was $y = (3.515 \cdot 10^{-6})x$, where y is concentration of isoxanthopterin (μM) and x is peak area, and the correlation coefficient was 0.99. The recovery was 97% at all concentrations. A representative chromatogram is shown in Fig. 3. The peaks of pterin and isoxanthopterin were observed at 6.2 and 8.3 min, respectively. The area of the isoxanthopterin peak indicates that the concentration of isoxanthopterin in the reaction mixture was 40 nM after incubation for 4 h.

3.4. Identification of isoxanthopterin in reaction mixtures

The retention time and emission ratio were determined in order to identify isoxanthopterin in

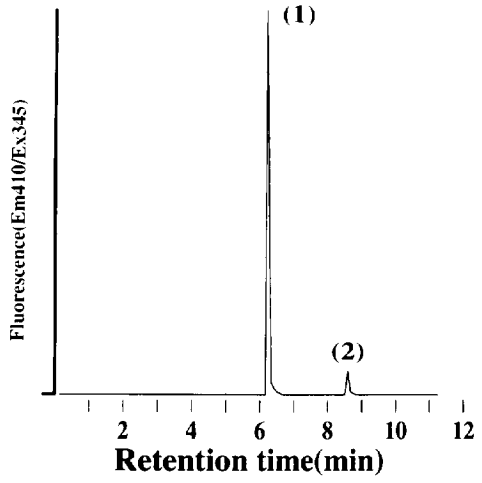

Fig. 3. Representative HPLC chromatogram of plasma reaction mixture after incubation for 4 h. Peaks 1 and 2 are pterin and isoxanthopterin, respectively. The concentration of isoxanthopterin in the reaction mixture was 40 nM.

reaction mixtures. Any deviation from the expected values of these parameters for isoxanthopterin may indicate other compounds eluting near to or simultaneously with isoxanthopterin. The retention time of authentic isoxanthopterin was 8.3 min. At an excitation wavelength of 345 nm, the 390/410 nm emission ratio of the peak in the reaction mixture with the same retention time as isoxanthopterin in standard solution was 0.66, identical to that of authentic isoxanthopterin. Allopurinol, a specific inhibitor of xanthine oxidase, was added to reaction mixtures at a final concentration of 0.3 μM; after incubation for 4 h, the amount of isoxanthopterin in the reaction mixture was below the detection limit, confirming that pterin is oxidized to isoxanthopterin in normal reaction mixtures by xanthine oxidase in plasma.

3.5. Effects of heparin and ethanol on plasma xanthine oxidase activity

For five control subjects, plasma xanthine oxidase activity 15 min after intravenous injection of heparin did not differ from that before heparin administration (14.8±2.5 vs. 14.8±2.8 pmol/ml/h, respectively). Similarly, for another group of five control subjects, plasma xanthine oxidase activity 30 and 90 min after ethanol ingestion (14.5±3.0 and 14.7±2.8 pmol/ml/

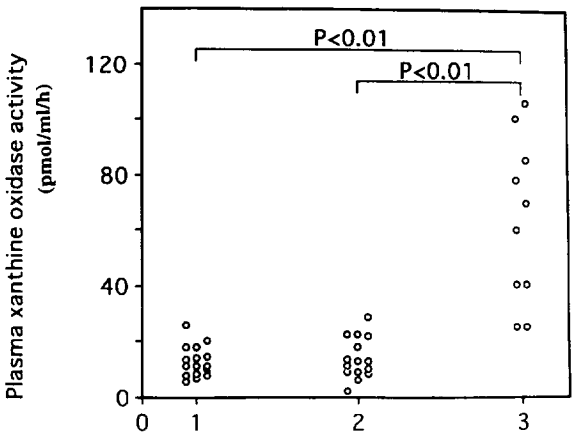

Fig. 4. Comparison of plasma xanthine oxidase activity among control subjects (1), patients with gout (2) and patients with hepatitis C virus infection (3).

h, respectively) did not differ from that before ethanol ingestion (14.8±3.0 pmol/h/ml)

3.6. Plasma xanthine oxidase activity in normal subjects, patients with gout or hepatitis C virus infection and a patient with von Gierke's disease

The activity of xanthine oxidase in plasma was 12.8±5.7 pmol/ml/h in healthy subjects, 14.0±7.3 pmol/ml/h in patients with gout, and 63.3±28.8 pmol/ml/h in patients positive for antibodies to hepatitis C virus with increased AST and ALT (Fig. 4). Plasma xanthine oxidase activity in the patients with hepatitis virus C infection was significantly higher than that in the healthy controls and in the patients with gout. Plasma xanthine oxidase in a patient with von Gierke's disease was 280 pmol/ml/h.

4. Discussion

A sensitive HPLC method for measuring the activity of xanthine oxidase with pterin as a substrate was previously described [10]. However, xanthine oxidase activity in human plasma was too low for measurement by this method. Because human plasma xanthine oxidase is stable, we prolonged the incubation time at optimal pH (9.0) to enhance the sensitivity of the assay. In addition, we increased the substrate concentration because plasma xanthine and hypoxanthine may competitively inhibit the production of isoxanthopterin by xanthine oxidase. The physiological concentration of uric acid in plasma was previously shown to inhibit the activity of xanthine oxidase [7]. However, because uric acid at a final concentration of 180 μM in the mixture did not affect plasma xanthine oxidase activity in the present study, uric acid at concentrations up to 0.9 mM in plasma should not affect plasma xanthine oxidase in our assay. We have also shown that high concentrations of hypoxanthine in the reaction mixture inhibit the activity of xanthine oxidase in plasma; thus, plasma should be separated immediately from whole blood because hypoxanthine leaks from erythrocytes into plasma if the separation is markedly delayed. In contrast, the concentration of xanthine in plasma did not increase substantially if the separation of serum from whole blood was delayed. With the exception of patients with xanthinuria or those taking allopurinol, the concentration of xanthine in plasma should therefore rarely inhibit the activity of xanthine oxidase.

With the use of polyclonal antibodies to human xanthine oxidase, Adachi et al. [11] showed that the concentration of xanthine oxidase in human plasma after injection of heparin was twice that before heparin injection. These researchers suggested that xanthine oxidase binds to the polysaccharide chains of heparin-like proteoglycans on the endothelial cell membrane and that the increase in the plasma concentration of xanthine oxidase after injection of heparin results from displacement of enzyme bound to heparin sulphate located on the endothelial cell surface. However, in the present study, we did not detect a difference between the activity of xanthine oxidase in plasma before intravenous injection of heparin and that after heparin injection, suggesting that xanthine oxidase dislodged from the endothelial cell surface by heparin is not active and does not play a role in the xanthine oxidase-mediated production of oxygen radicals. Peritoneal administration of ethanol in rats increases the concentration of xanthine oxidase in plasma without a concomitant increase in AST and ALT in plasma [12], indicating that plasma xanthine oxidase is a sensitive marker of liver damage. However, oral administration of ethanol (0.8 g/kg body weight) in the present study did

not affect the activity of xanthine oxidase in the plasma of normal subjects, although the acute effect of large amounts of ethanol and the chronic effect of this agent on human plasma xanthine oxidase activity remain to be determined.

Primary gout is a disorder of purine metabolism. Clinically, it is classified into types characterized by the overproduction of uric acid (overproduction type), the underexcretion of uric acid (underexcretion type), or both overproduction and underexcretion of uric acid (mixed type). Although its causes remain unknown, the release of enzymes that participate in purine catabolism, including xanthine oxidase, into plasma may be increased, especially in patients with gout of the overproduction type. However, we did not detect a significant difference in plasma xanthine oxidase activity between patients with gout and normal controls (Fig. 4). In contrast, previous studies [3–5] have shown that the activity of xanthine oxidase in serum is increased in patients with viral hepatitis. However, when these studies were performed, hepatitis C virus had not been discovered. We have now shown that plasma xanthine oxidase activity in patients with hepatitis C virus infection is significantly higher than that in controls (Fig. 4). We also showed that plasma xanthine oxidase activity was markedly increased in a patient with glucose 6-phosphatase deficiency having hepatic adenomas. Therefore, it is possible that the high plasma enzyme activity in this patient is attributable to glucose-6-phosphatase deficiency-related hepatic adenomas which were confirmed histologically. However, to clarify this possibility, determination of plasma xanthine oxidase activity in many patients with von Gierke's disease is needed.

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