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Improved method for measurement of human plasma xanthine oxidoreductase activity

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Abstract

The XOR activity in human plasma was measured by quantifying the XOR-derived uric acid (UA) in plasma using the high-performance liquid chromatography (HPLC) equipped with a UV detector. Chromatographic separation consisted of the mobile phase (a mixture of 0.1% trifluoroacetic acid in Milli-Q water and 0.085% trifluoroacetic acid in acetonitrile in a mix ratio of 99:1) running through a Zorbax StableBond SB-C_{18} column at a flow-rate of 1 ml/min. Deproteinization with heat-treatment of plasma samples after the reaction was used in the assay to avoid splitting of the UA and xanthine peaks caused by acid deproteinization that could interfere the accurate determination of human plasma XOR activity in our case. Based on the examination of the dependence of XOR activity on added amounts of xanthine and reaction times, the amount of xanthine and reaction time for XOR activity assay were determined to prevent the errors caused by the limiting effect of substrates and plateau phase of the reaction. Using this method, human plasma XOR activities of 25 healthy people were measured. The average human plasma XOR activity was 2.1 ± 0.8 ($\times 10^{-3}$ U/ml).

Keywords: Xanthine oxidoreductase; Enzymes

1. Introduction

Xanthine oxidoreductase (XOR) is a rate-limiting enzyme in purine metabolism and produces uric acid (UA) as the end product in humans. In mammals, XOR exists in two convertible forms, xanthine dehydrogenase (XDH, EC 1.1.1.204) and xanthine oxidase (XO, EC 1.1.3.22). XDH transfers the reducing equivalents generated by the oxidation of substrates to NAD^+, whereas XO transfers them to oxygen [1]. XOR activity represents the total activity of both forms [2,3]. Studies have indicated important roles of XOR in clinical disorders including purine metabolism-related disorders such as xanthinuria, gout and hyperuricaemia [4–6] and the oxidative tissue injury-associated disorders such as stroke and heart attack [7,8]. The release of XOR into circulation from injured tissues such as the injured liver has been demonstrated in animals [9,10] as well as in humans [11–13]. Thus, the XOR activity in plasma potentially can be used as a sensitive marker of oxidative tissue injury.

In contrast to the importance of plasma XOR in studying XOR-associated clinical disorders, the determination of human plasma XOR activity has been

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a difficulty due to the rather low activity of XOR in humans in relative to rodents [14]. To accurately determine the XOR activity, several factors usually have to be considered in developing appropriate analytical assays. These factors include choosing the chemicals representing the end products or substrates of XOR as physiological and specific as possible and the assay being as sensitive as possible. To date, several methods including fluorometric, spectrophotometric and radiometric and ELISA assays have been developed [11,12,15,16]. Among them, the high-performance liquid chromatography (HPLC) with spectrophotometrical detection of XOR-derived UA is commonly used. This method provides a high sensitivity associated with an efficient chromatographic separation of UA from other similar chemicals and has been used to determine the plasma XOR activity [12].

In an effort to study the function of plasma XOR in clinical disorders, we attempted to measure human plasma XOR activities following the previous studies [11,12]. In the study, we found that the method for measurement of human plasma XOR activities could be improved by modifying several detailed procedures including HPLC conditions, deproteinization methods, amounts of substrates used in reaction mixtures as well as reaction times. Using the modified method, the plasma XOR activities of 25 healthy people were measured.

2. Experimental

2.1. Chemicals and stock solutions

Uric acid (UA), xanthine and allopurinol were obtained from Sigma (USA). Uricase was obtained from Boehringer Mannheim (Germany). Acetonitrile (HPLC grade) was obtained from Fluka Chemika (Switzerland). Trifluoroacetic acid and perchloric acid were HPLC grade and obtained from Baker (USA). Stock solutions were prepared by dissolving the chemicals in ddH_2O.

2.2. Apparatus and technique

A HP1100 HPLC system (Agilient Company) equipped with UV detector and an injector with a 20-μl loop was used. The wavelength was set at 280 nm with a reference at 360 nm. The mobile phase was composed of 0.1% trifluoroacetic acid in Milli-Q water and 0.085% trifluoroacetic acid in acetonitrile with a mix ratio of 99:1 (pH 2.0–2.5). The flow-rate of the mobile phase was 1.0 ml/min and the column temperature was 50 °C. A Hypersil ODS column (250×4.0 mm, 5.0-μm particle size, Agilient Company) was used to examine the effect of perchloric acid on peaks of UA and xanthine in HPLC analysis. A Zorbax StableBond SB-C_{18} column (80 Å, 250×4.6 mm, 5.0-μm particle size, Agilient Company) was used for the measurement of human plasma XOR activities.

2.3. Blood sample collection

Blood samples were collected from healthy people in tubes containing EDTA and then immediately centrifuged for 10 min at 1500 *g*, 4 °C to collect the plasma. The plasma samples were stored at −20 °C until being thawed for use.

2.4. Plasma XOR activity assay

2.4.1. Basic procedures for plasma XOR activity assay

Reaction mixtures containing the plasma and added xanthine were incubated at 37 °C for an appropriate time followed by deproteinization to stop the reaction and remove the proteins. After centrifugation, the supernatants were saved for HPLC analysis of UA. The deproteinization method, the amount of xanthine and reaction time were chosen for XOR activity assay based on the results obtained from the following experiments.

2.4.2. Methods for deproteinization of plasma proteins

For acid precipitation of plasma proteins, perchloric acid was added to the plasma samples at a final concentration of 10% (v/v). As a control, perchloric acid was replaced with the same volume of ddH_2O. After mixing with a brief vortex, the samples were centrifuged at 13 000 rpm for 15 min and supernatants were then saved for HPLC analysis.

To examine the effect of perchloric acid on peaks of xanthine, perchloric acid was added to 1 m*M*

xanthine solution at a final concentration of 10 or 30% (v/v). As a control, perchloric acid was replaced with the same volume of ddH_2O. After being mixed briefly, the samples were directly used for HPLC analysis.

In the present study, the deproteinization method chosen for human plasma XOR activity assay was a heating method, which was performed by boiling the plasma samples for 10 min after the reaction to stop the reaction and deproteinize the plasma samples. The heat-treated samples were then centrifuged for 15 min at 13 000 rpm. The supernatants were saved for HPLC analysis.

2.4.3. Dependence of UA production on added amounts of xanthine

To determine the amount of xanthine required for XOR activity assay, the dependence of UA production on added amounts of xanthine was studied by determining the UA amounts produced in reaction mixtures containing different concentrations (0.1, 0.3, 0.5, 0.7 and 0.9 m*M*) of xanthine. In the experiments, the same volume (100 μl) of aliquots from the same plasma sample was added to reaction mixtures containing different amounts (100, 300, 500, 700 and 900 μl) of 1 m*M* xanthine solution. The ddH_2O was added to adjust the total volume equal to 1 ml for each reaction mixture. The reaction mixtures were incubated at 37 °C for 60 min. A reaction mixture containing 100 μl plasma and 900 μl ddH_2O was used as a control. After the incubation, the mixtures were heat-treated and centrifuged as described above to save the supernatants for HPLC analysis.

2.4.4. Dependence of UA production on reaction times

The reaction time required for XOR activity assay was determined by examining the UA production at various reaction times. The reaction mixtures containing 100 μl plasma from the same plasma sample and 0.9 m*M* xanthine were incubated at 37 °C for different times (5, 10, 20, 30, 40, 50 and 60 min). As a control, xanthine was replaced with 900 μl ddH_2O. After the incubation, the mixtures were heat-treated and centrifuged as described above to save the supernatants for HPLC analysis. The XOR-derived UA (nmol) was the difference of UA amounts between the control and test samples (0.9 m*M* xanthine). Results of three plasma samples (each with triplicate analyses) were reported.

2.4.5. Effects of allopurinol and uricase on UA production

An XOR inhibitor, allopurinol (1 μl of 100 m*M* allopurinol solution) was added to the reaction mixture containing 100 μl plasma and 900 μl 1 m*M* xanthine (0.9 m*M*). After incubation at 37 °C for 60 min, the mixtures were heat-treated and centrifuged as described above to save the supernatants for HPLC analysis and compared with reaction mixture of the same plasma sample without addition of allopurinol.

To confirm the UA peaks, after the reaction and sample processing, the supernatants of the reaction mixture containing 100 μl plasma and 900 μl 1 m*M* xanthine were incubated with uricase (0.1 mg/ml) for 2 h at 37 °C before being applied for HPLC analysis. After the incubation, the supernatants were subjected to HPLC analysis.

2.4.6. Assay of XOR activity in plasma samples from 25 healthy people

Plasma XOR activities of 25 healthy people were assayed. For each sample, 100 μl of the plasma was used to react with 900 μl of 1 m*M* xanthine (as a test) or 900 μl ddH_2O (as a control). The reaction mixtures were incubated at 37 °C for 20 min followed by boiling for 10 min to stop the reaction. The samples were centrifuged at 13 000 rpm for 15 min to remove the proteins and save the supernatants for HPLC analysis of UA. Standard UA dissolved in ddH_2O was used to establish an external standard curve by plotting the areas of UA peaks versus UA concentrations. The areas of UA peaks in plasma samples were converted to UA concentrations using the established external calibration curve. The UA concentration of the control sample was considered as an endogenous or a background UA and subtracted from the UA concentration of the test sample. A unit of XOR specific activity was presented as 1 U=1 μmol XOR-derived UA/min.

2.5. Minimum detectable concentration, calibration curve, variability and recovery

The minimum detectable concentration for UA was defined as a peak of UA produces three times of baseline noise ($S/N=3$). An external calibration curve was prepared by triplicate analyses of standard UA solutions with concentrations ranging from 0.5 to 500 μM. To examine the variability of XOR activity assay produced by sample processing, the XOR activity of the same plasma sample was assayed by five separate experiments with the same processing procedure as described above. Each separate experiment was subjected to triplicate analyses. To examine the UA recovery efficiency associated with the sample processing, three different amounts (1, 10 and 100 μM) of external standard UA were added to the plasma samples followed by the same processing procedures as described above. Then, UA amounts recovered from the samples after the processing were measured. A total of five separate plasma samples were used to examine the UA recovery from three different amounts of external UA added in triplicate. The amount of UA in the samples without addition of external UA was determined as a background. The UA recovery efficiency was calculated using an equation:

$$\text{Recovery efficiency} = (A-B)/C$$

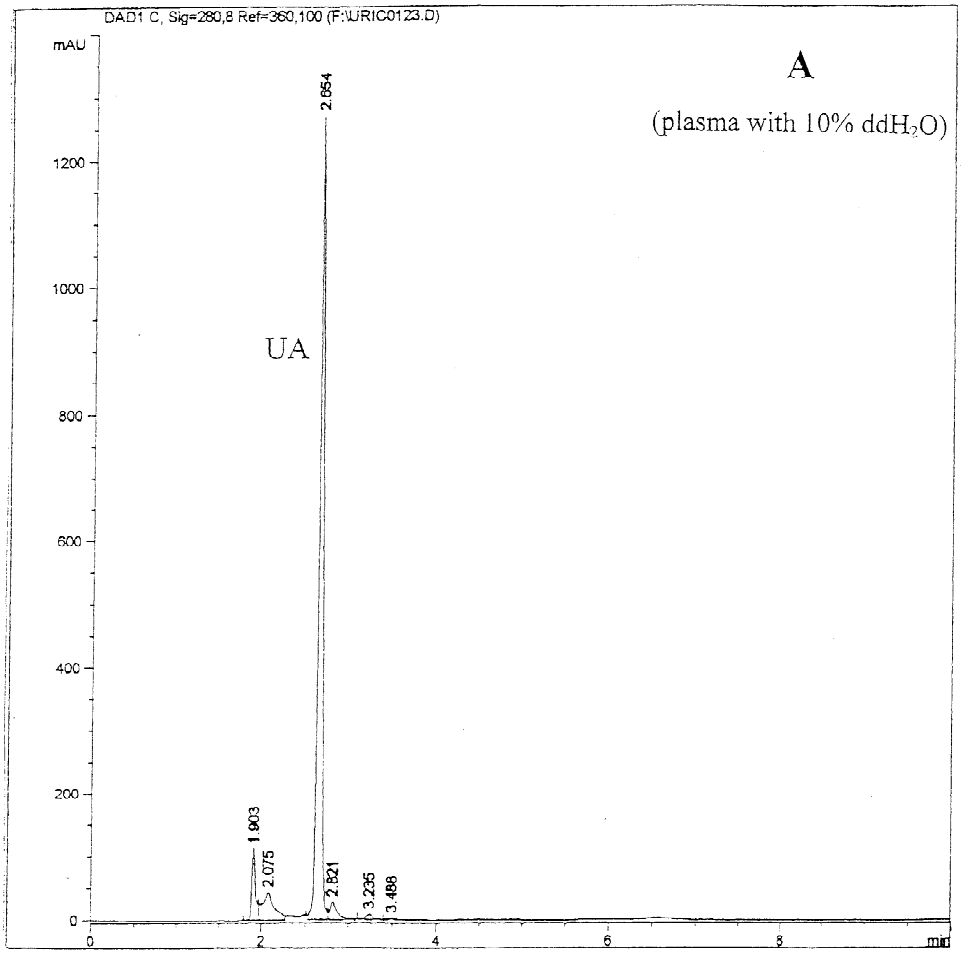


Fig. 1. Perchloric acid treatment alters the peaks of plasma UA and xanthine. The peaks of UA in plasma without (A) and with (B) 10% (v/v) perchloric acid treatment. The peaks of xanthine without (C) and with 10% (v/v) (D) or 30% (E) perchloric acid treatment.

Where *A* is amount of UA in a plasma sample with addition of external UA; *B* is amount of UA in the same sample without addition of external UA; and *C* is the amount of external UA added.

3. Results and discussion

3.1. Mobile phase and HPLC column

In previous studies, the acidic mobile phases, generally a combination of acidic buffer such as phosphate buffer with acetonitrile or methanol (pH 3.0–5.0), have been successfully applied in HPLC analysis of UA [11,15,17–22]. In the present work, we examined several acidic mobile phases similar to those reported previously and the acid tolerance of several types of HPLC columns. The results showed that the more acidic the mobile phases, the better the separation of UA (data not shown). Finally, a strong acidic mobile phase (a mixture of 0.1% trifluoroacetic acid in Milli-Q water and 0.085% trifluoroacetic acid in acetonitrile in a mix ratio of 99:1, pH 2.0–2.5) was chosen for our experiments. At the same time, we chose a Zorbax StableBond SB-C_{18} column for HPLC analysis of UA because it is tolerant to the mobile phases as acidic as pH 1. In combination with the acidic mobile phase described above, this column provided a high efficiency in

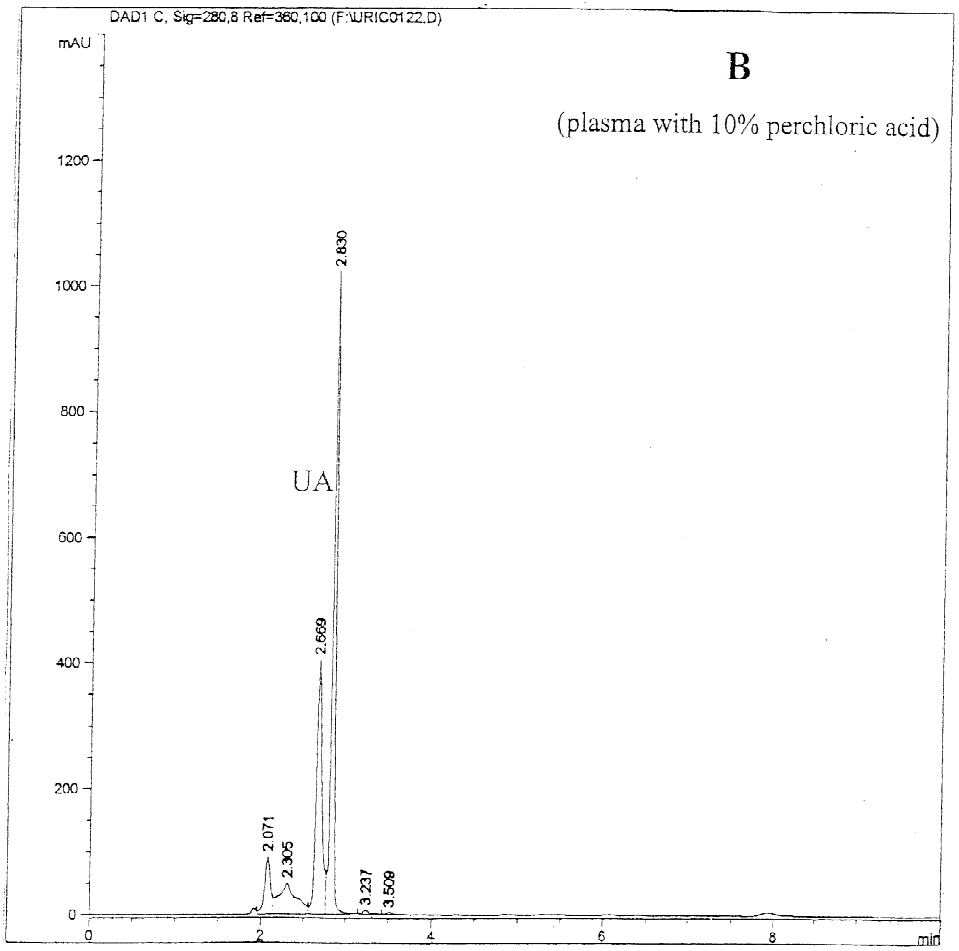


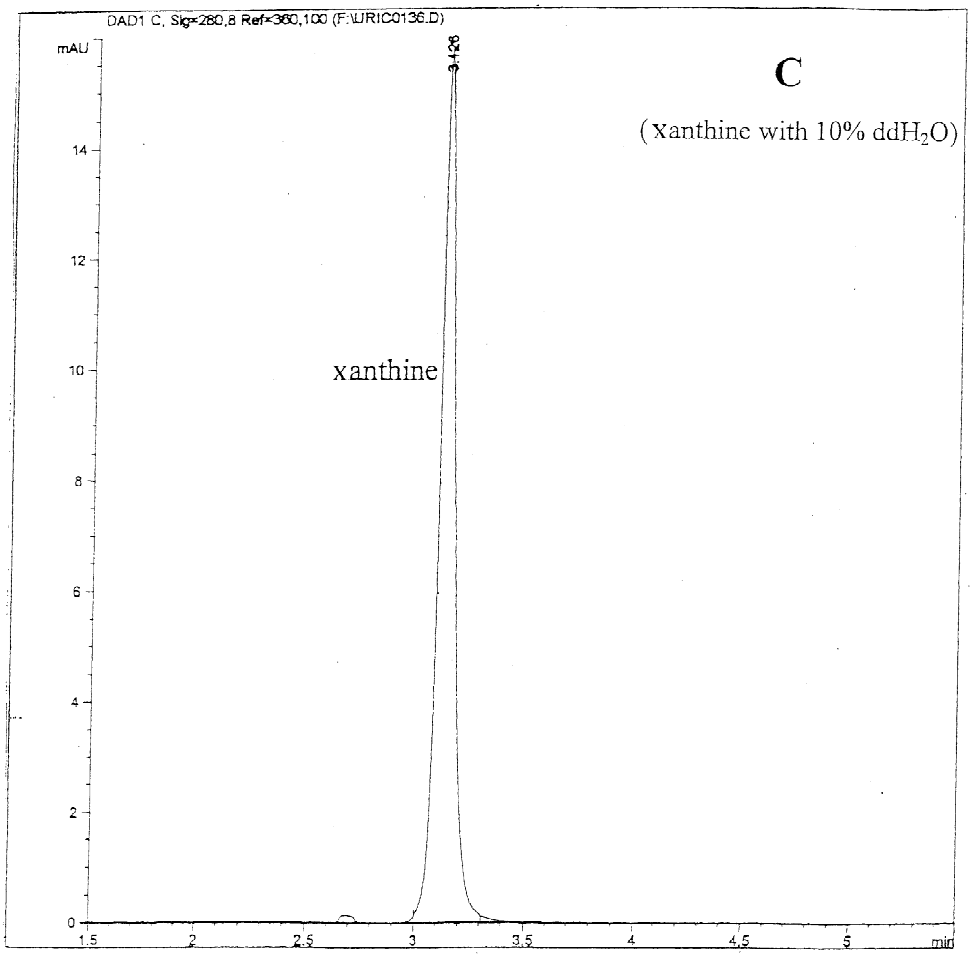
Fig. 1. (*continued*)

separation of UA and was used in plasma XOR activity assay.

3.2. Deproteinization methods for plasma XOR activity assay

To avoid the precipitation of plasma proteins in acidic mobile phases, plasma samples were deproteinized before HPLC analysis. Acid precipitation using acids such as perchloric acid and trichloroacetic acid was the most widely used method for plasma protein removal in previous studies [17,18,23]. However, our results showed that, under the conditions we used, perchloric acid caused the splitting of UA and xanthine peaks as shown in Fig. 1. Compared to Fig. 1A, Fig. 1B shows that treatment with 10% perchloric acid causes the splitting of plasma UA peaks. Moreover, as shown in Fig. 1C–E, perchloric acid also causes the splitting of xanthine peaks. One of the split xanthine peaks had a retention time similar to that of UA, causing difficulty in separation of UA peaks from xanthine peaks. Acids other than perchloric acid also had similar effects (data not shown). Because the assay of XOR activity was based on the accurate determination of UA produced from xanthine by XOR, the acid precipitation of plasma proteins appeared unsuitable to our HPLC analytical system for XOR activity assay.

Two other conventional methods for deproteiniza-



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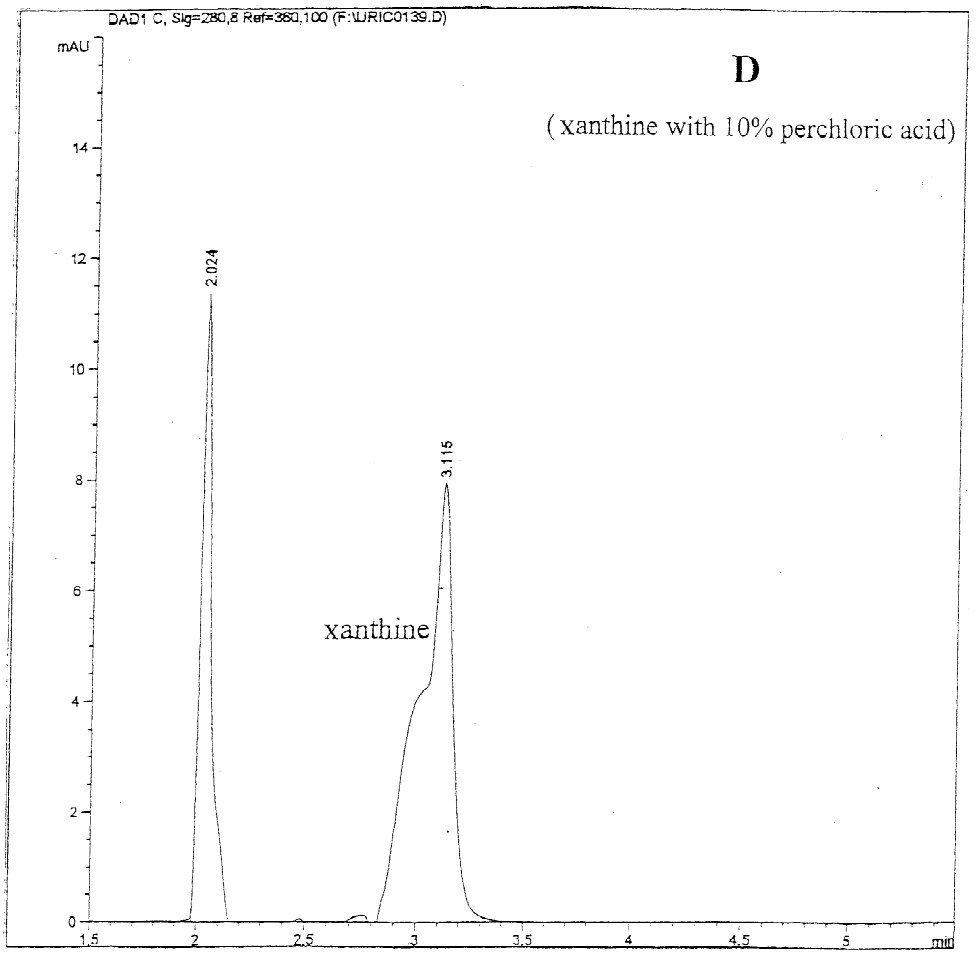

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tion, phenol–chloroform extraction and heating, were also examined in the present study. We found that the extraction of plasma samples with phenol–chloroform caused a great loss of UA although the proteins were almost completely removed by the extraction (data not shown). Therefore, we could not accept the phenol–chloroform extraction as a deproteinization method for the measurement of plasma XOR activity. In comparison, we demonstrated that heating of samples after reaction at 100 °C for 10 min followed by centrifugation effectively removed proteins from samples without causing alteration of UA or xanthine peaks or the loss of UA. Therefore, the heating method was chosen in the present study for deproteinization of the samples.

3.3. The amount of xanthine used for XOR activity assay

To accurately measure XOR activity, the limiting effect of amount of substrate (xanthine) on XOR activity should be avoided. In the present study, we examined the dependence of UA production on the amount of xanthine. As shown in Fig. 2, the peak area of UA was 182.28 mAU·s in the control (Fig. 2A) and increased to 227.11 mAU·s in the presence of 0.1 m*M* xanthine (Fig. 2B) and to 242.76 mAU·s in the presence of 0.3 m*M* xanthine (Fig. 2C). The peak area of UA was constant when the xanthine concentration in reaction mixtures was greater than 0.3 m*M* (Fig. 2D–F). In the present study, the XOR

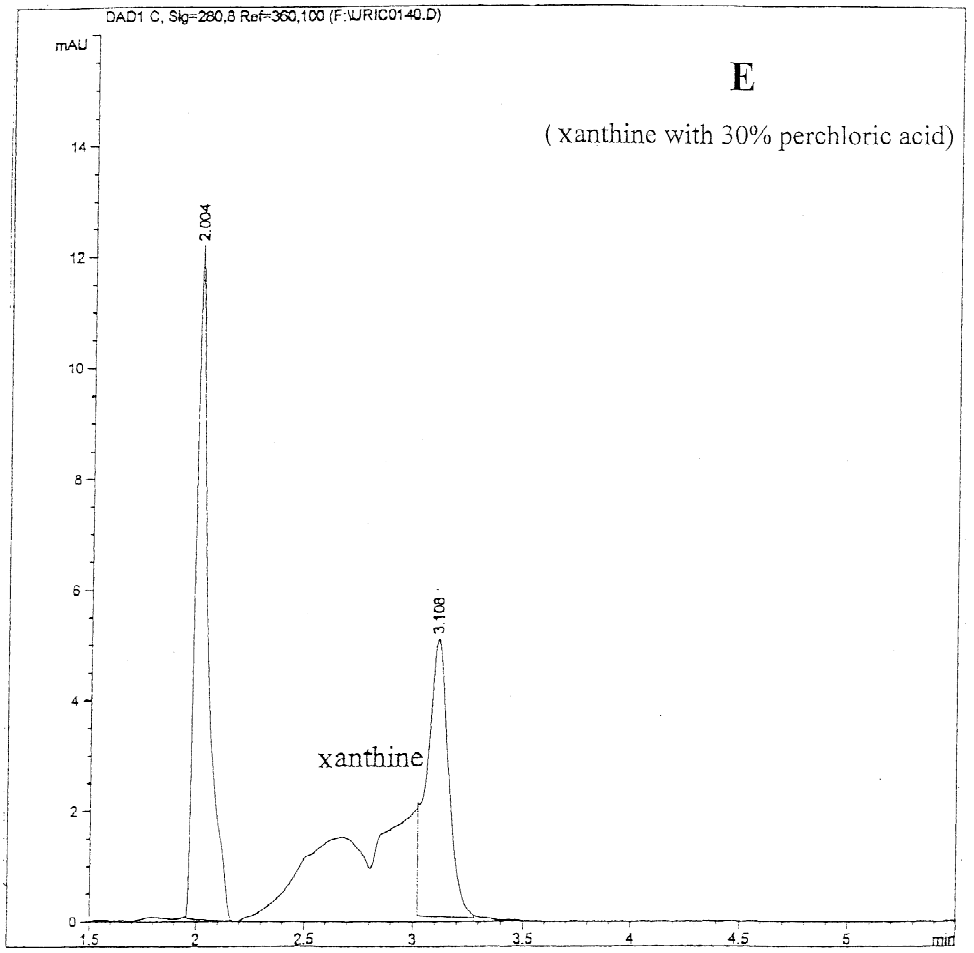


Fig. 1. (*continued*)

activity was determined in the presence of 0.9 m*M* xanthine to avoid the limiting effect of xanthine amounts on XOR activity assay.

3.4. The reaction time for XOR activity assay

For accurate measurement of XOR activity, the other issue to be considered is to choose a reaction time that is located within the linear reaction phase, not the plateau phase. As shown in Fig. 3, the UA increase (XOR-derived UA) in the reaction mixtures at different reaction times ranging from 5 to 60 min were examined. The result showed that UA production increased linearly with reaction times from 5 to 30 min. In the present study, a 20-min reaction time was applied for XOR activity assay.

3.5. Characterization of UA produced in reaction mixtures

An XOR inhibitor, allopurinol, was used to determine whether the UA produced in reaction mixtures was XOR-derived UA. As shown in Fig. 2, in the absence of allopurinol, the peak area of UA in the reaction mixture containing 0.9 m*M* xanthine was 242.73 mAU·s. In comparison, in the presence of 100 μ*M* allopurinol, the peak area of UA was 186.52 mAU·s (Fig. 2G), which was similar to that of the control (no added xanthine, Fig. 2A). The result

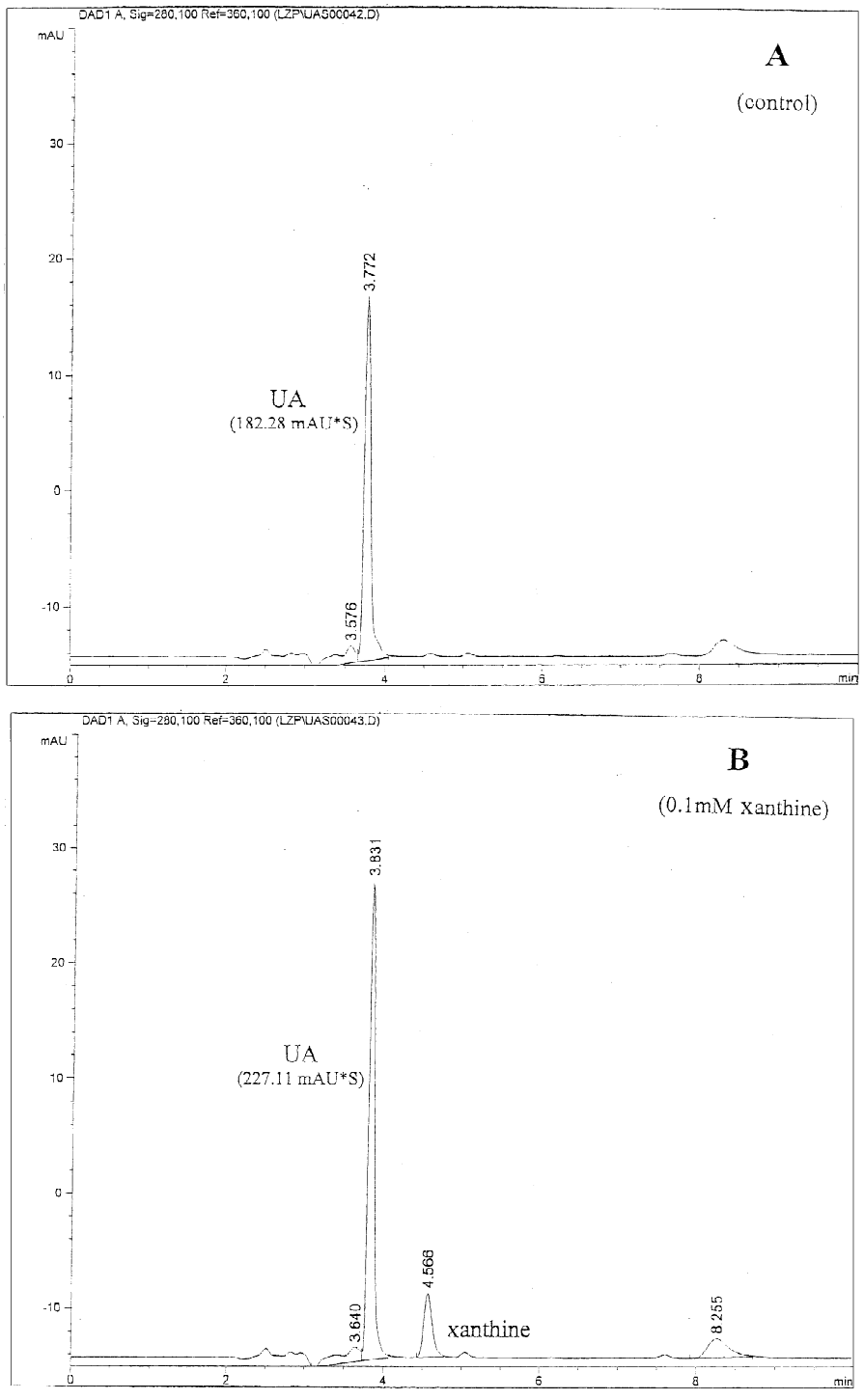
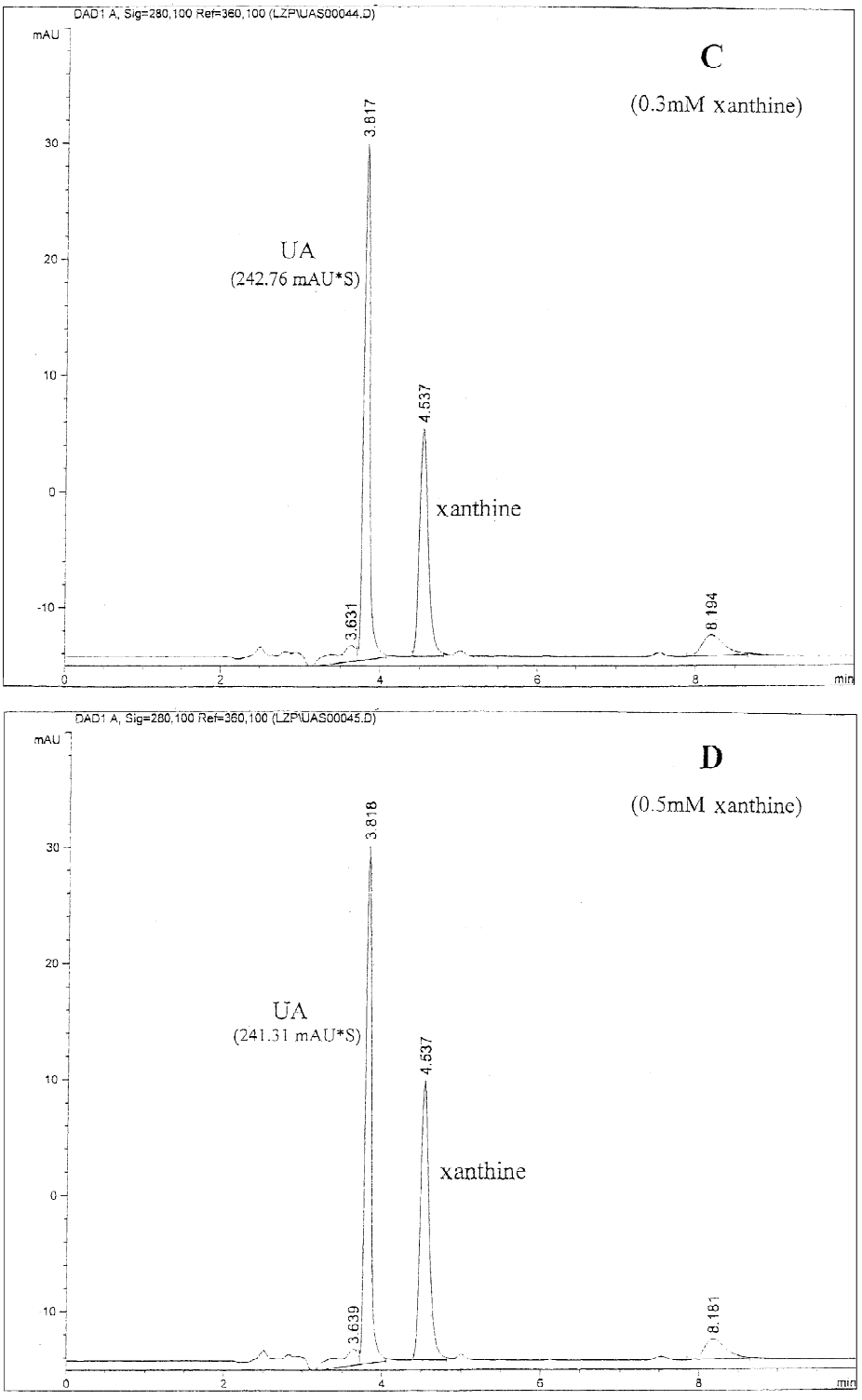


Fig. 2. Dependence of UA production on amounts of xanthine added in reaction mixtures. (A) Control, no added xanthine; (B) 0.1 m*M* xanthine; (C) 0.3 m*M* xanthine; (D) 0.5 m*M* xanthine; (E) 0.7 m*M* xanthine; (F) 0.9 m*M* xanthine; (G) 0.9 m*M* xanthine and 100 μ*M* allopurinol; (H) 0.9 m*M* xanthine with uricase treatment.



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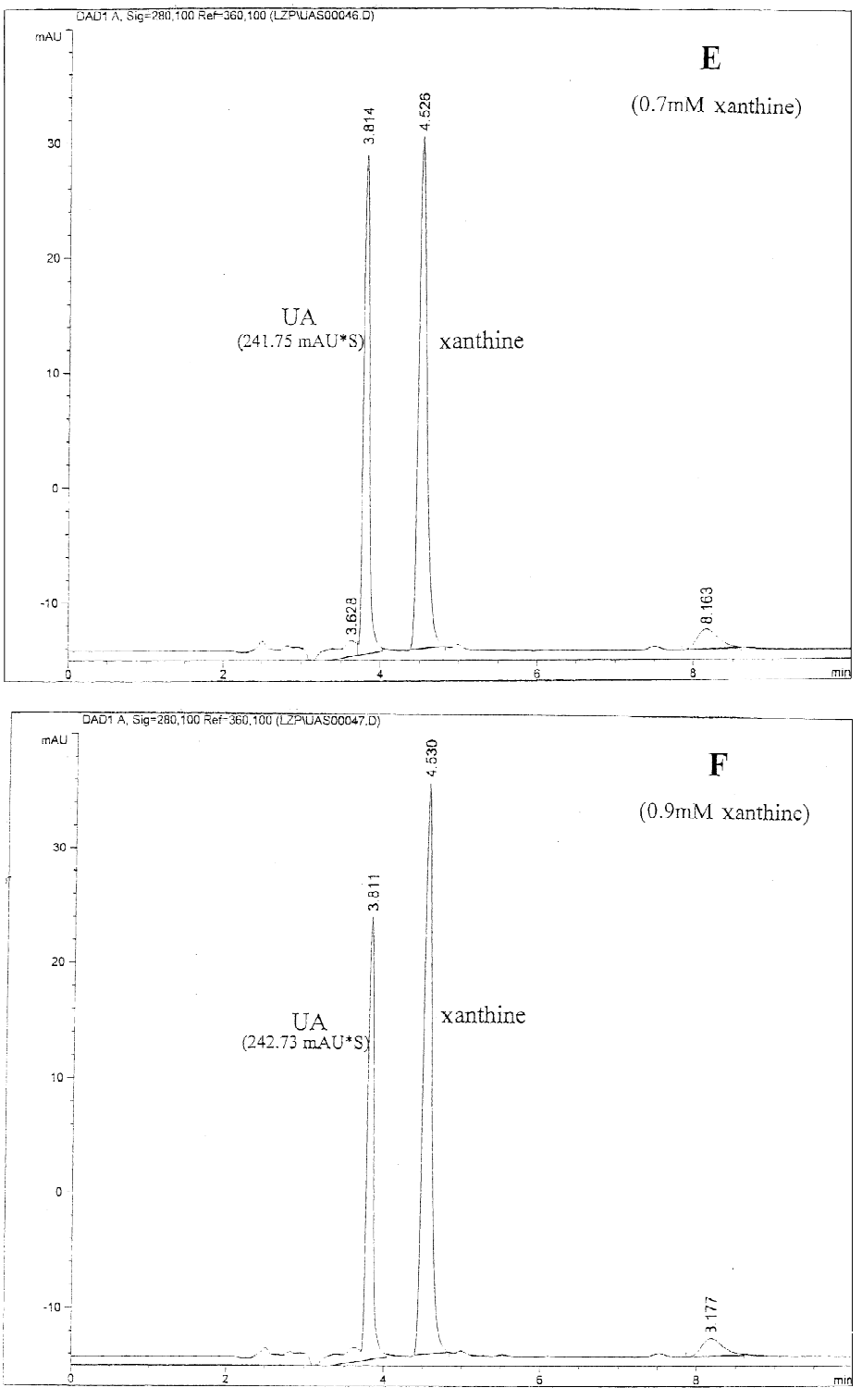

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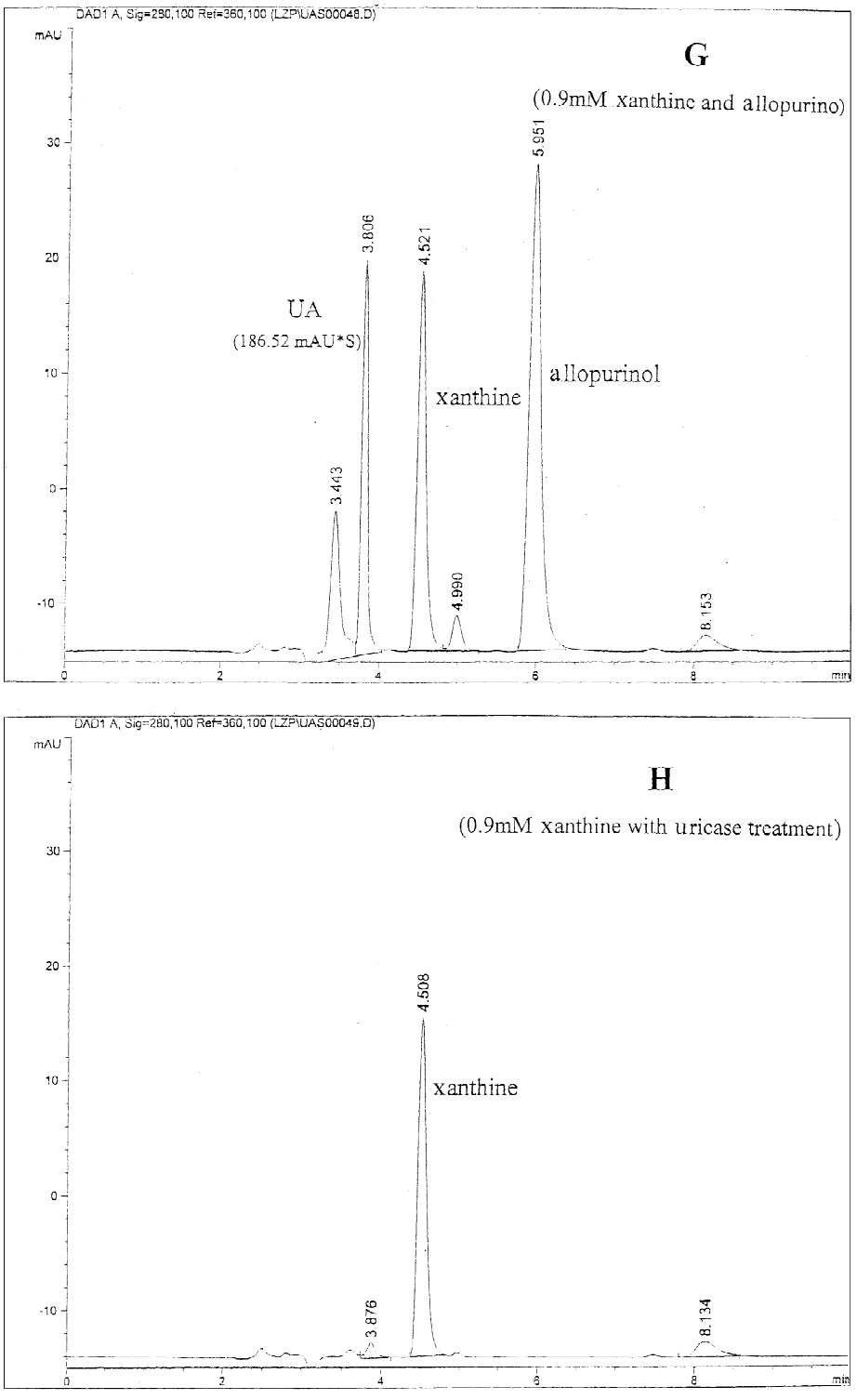

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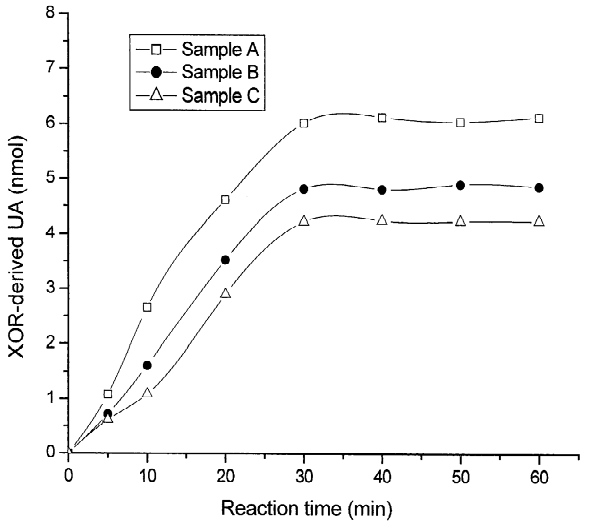

Fig. 3. Dependence of UA production on reaction times. The difference of amount of UA between the samples without (control) and with 0.9 m*M* xanthine represents XOR-derived UA. The figure summarizes the results of three independent experiments (each with triplicate analyses).

showed that the production of UA in reaction mixture was inhibited by allopurinol, indicating that UA produced in reaction mixtures is XOR-derived UA. Besides, the peak of UA was almost completely diminished by treatment with uricase for 2 h, demonstrating that the peak represents UA, not the UA analogs (Fig. 2H). Taken together, the results demonstrated that the difference of UA peak areas between the plasma incubated with xanthine and the plasma incubated with ddH_2O (control) represents the XOR-derived UA.

3.6. Minimum detectable concentration, calibration curve, variability and recovery efficiency

In the present study, we showed that the minimum detectable concentration of UA, an indicator for the sensitivity of assay, was 0.75 ng. In addition, the UA concentrations ranging from 0.5 to 500 μ*M* showed a linear relation with UA peak areas ([UA] (μ*M*) = 0.119 × Peak area (mAU·s) with a regression coefficient of 0.998 and a standard error of 1.877 for the slope. The intercept of the calibration curve was 1.095. The average variability of XOR activity assay was 1.3–2.5%. The recovery efficiency of UA in the XOR activity assay procedures was 92.6–97.3%.

3.7. Assay of XOR activity in plasma samples from 25 healthy people

As shown in Table 1, the XOR activity in human plasma can be measured based on the differences between the amounts of UA before and after the reaction. The average UA background concentrations of 25 samples were 193±31 μ*M*. The UA concentration in human serum reported previously was 2.7–7.3 mg/dl [22]. Considering that the molecular mass of UA is 190.1, our result is close to those previously reported. In the present study, the average human plasma XOR activity was 2.1±0.8 ($\times 10^{-3}$ U/ml). Up to now, study of the measurement of human plasma XOR activity is limited [11,15]. Tan et al. reported that human plasma XOR activity was 0.12±0.12 μU/mg protein for seven adult healthy volunteers and 1.16–3.77 μU/mg protein for a patient with aortic aneurysm [11]. In comparison to

Table 1
The concentration of UA and XOR activity in human plasma

Samples	UA (background, μ*M*)	UA (after reaction, μ*M*)	XOR activity ($\times 10^{-3}$ U/ml)
1	227	298	3.6
2	216	250	1.7
3	181	214	1.6
4	192	225	1.6
5	159	203	2.2
6	246	295	2.4
7	242	271	1.4
8	194	219	1.2
9	161	210	2.5
10	201	274	3.6
11	168	227	2.9
12	223	247	1.2
13	206	241	1.8
14	199	270	3.5
15	260	286	1.3
16	173	206	1.6
17	161	215	2.7
18	204	247	2.1
19	163	188	1.3
20	134	174	2.0
21	192	226	1.7
22	176	237	3.1
23	154	193	1.9
24	182	226	2.2
25	214	266	2.6
Average	193±31		2.1±0.8

that report, the human plasma XOR activity obtained in our study is slightly higher. This difference may be attributed to several factors such as the relatively longer reaction time (60 min) and the acid precipitation for protein removal used in their study. The human plasma XOR activity reported by Yamamoto et al. [15] is not comparable to the present study because they used pterin as the substrate and defined the XOR activity as picomoles of isoxanthopterin produced per hour per milliliter (pmol/h per ml).

In brief, we improved the method for measurement of human plasma XOR activity by choosing appropriate HPLC condition, deproteinization method, substrate amount and reaction time. The HPLC conditions applied in the present study have not been reported previously. In addition, the present study demonstrates that deproteinization with heat-treatment of plasma samples after the reaction effectively avoids the acidic deproteinization-caused alteration of UA and xanthine peaks which occurred in our case. Based on the examination of the dependence of XOR activity on added amounts of xanthine and reaction times, we chose the amount of xanthine and reaction time for the assay to avoid the errors resulting from the limiting effect of substrates and the plateau phase of the reaction. Moreover, the present method is more physiological in terms of the substrates of the enzyme [11] compared to use of xanthine analogs as substrates [15].

The alteration of XOR activity in human plasma has been recognized as a potential indicator of clinical disorders associated with XOR dysfunction. Thus, the measurement of human plasma XOR activity not only benefits the basic research of XOR in humans, but also the pathological functions of XOR in XOR-relevant clinical disorders. In conclusion, the present work provides a useful method for studying the functions of XOR in humans either in clinical or basic research settings.

4. Notation

ddH_2O, distilled deionized H_2O
HPLC, high-performance liquid chromatography
mAU·s, milli-absorption unit·s
UA, uric acid
XOR, xanthine oxidoreductase

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