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An Improved, Highly Sensitive HPLC-based Method for Determining Uric Acid Levels in Microliter Plasma Volumes

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Abstract

Here, we describe quantitative analysis of plasma uric acid (UA) levels using an improved HPLC-UV method, which provides highly sensitive analysis using microliter volumes of plasma. Phosphoric acid aqueous solution (pH 1.8) was used as the mobile phase. The quantitative analysis of oxypurines in plasma using a polymeric-ODS with high-carbon content (*ca.*17 %) column ($\phi 4.6 \times 250$ mm) was accurate and reproducible. The reproducibility of the UA analysis had a determination limit of 0.14mg/dL and a coefficient of closing variation of 0.98 %.

The results by our method confirmed that the plasma UA levels were increased in mice with oxonic acid-induced hyperuricemia compared to normal mice, and that allopurinol reduced the plasma UA levels in these mice. This method allows the measurement of plasma uric acid levels using small plasma samples.

INTRODUCTION

A large number of patients suffer from gout or hyperuricemia. For diagnosis of hyperuricemia and to facilitate development of treatments for hyperuricemia, a sensitive uric acid (UA)

analysis method is necessary. The HPLC-UV method for UA detection in human plasma is used to determine the effectiveness of remedies for gout, and requires a minimum plasma volume of 150-300 μL ¹⁾. Current optimized assays, such as the Enzyme-UV-VIS method, are sensitive to errors caused by hemolysis and/or interfering substances, and require a minimum plasma (serum) volume of 300 μL ¹⁾. The guidelines for this assay require that one sample be measured continuously in triplicate^{2a,b)}, which necessitates a final total serum volume < 150 μL (equivalent to *ca.* 250-300 μL blood)³⁾. The collection of blood from the abdominal vein, and the anesthesia this requires, cause stress to small, experimental animals such as mice and rats.

Consequently, it is necessary to develop a highly sensitive and precise assay for UA, xanthine, allopurinol, and oxipurinol in plasma that requires fewer invasive surgical procedures on experimental animals. We have been developing methods for measuring compounds in small quantities of plasma that can be collected from a mouse-tail vein without anesthesia. This is now possible due to development of a UA assay that allows the use of microliter volumes of plasma.

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METHODS

Chemicals and Instruments

The following chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan): allantoin (All), allopurinol (AP), hypoxanthine (Hyp), oxipurinol (OP), potassium oxonate, xanthine (Xan), UA, and standard UA solution (10 mg/dL, Uric Acid-TEST WAKO, calibrated using National Institute of Standards and Technology; SRM913a). Xanthine oxidase from buttermilk was purchased from Calbiochem (EMD Biosciences, Darmstadt, Germany). Uricase from yeast was obtained from Oriental Yeast (Tokyo, Japan). Enzymatic reactions were conducted in a Pasorina Incubator PI-301 (Matsuura Seisakusyo, Tokyo, Japan). Ultraviolet and visible absorption (UV-VIS) spectra of oxypurines and plasma samples were measured using a spectrometer (V-630, JASCO, Tokyo, Japan).

The high-performance liquid chromatography (HPLC) system consisted of a PU-980 pump (JASCO, Tokyo, Japan), model U-620 column oven (Sugai, Tokyo, Japan), UV-VIS detector (UV-980, JASCO, Tokyo, Japan), and model C-R-8A integrator (Shimadzu, Kyoto, Japan).

HPLC analysis of UA and related compounds

We used reverse-phase HPLC to simultaneously quantify purine-pyrimidine metabolites, AP, and OP in plasma samples. The wavelength used by the HPLC-UV detector was determined from the spectra (200–300 nm, in H₂O, at 25 °C) of UA, Hyp, Xan, All, AP, and OP ($\lambda_{\max}$ 284, 248, 269, 200, 252 and 256 nm, respectively).

All standard aqueous solutions of UA and related compounds were prepared using reagent-grade chemicals in a vacuum drier at room temperature (for at least 48 h) and standard UA solution (10 mg/dL) as needed.

For HPLC and the stability test (temporal course) of the aqueous UA solution, a mixed test solution (AP, Hyp, OP, Xan, and UA at 0.7, 0.8, 1.6, 0.6, and 1.7 mg/dL, respectively) was used, immediately.

Each peak of interest was quantified based on its area; under the acidic conditions of the mobile phase (*ca.* pH 2.0), the effect of column conditions (temperature, *etc.*) on peak area and height is negligible^{2a,b)}. We determined that optimal separation (theoretical plates and durability performance) was achieved using a Cosmosil C18 AR-5 column (Nacalai Tesque, Kyoto, Japan) and elution with an aqueous phosphoric acid solution (30 mmol/L, pH 1.8).

Uric acid concentration in aqueous solution (stability test)

The stability test examined the temporal change in uric acid at 0, 1, 4, 72, 96, 120, and 168 h using the mixed test solution (AP, Hyp, OP, Xan, and UA).

Each sample was analyzed in triplicate. The evaluate samples were preserved in an air-tight glass container at room temperature (8–20 °C).

Identification of UA in plasma

Uric acid and Xan were identified in plasma using Kuroiwa methods^{5, 6)}. Briefly, xanthine oxidase (XO, 0.084 U, 10 μ L), uricase (UC, 0.075 U, 5 μ L), or water was added to pooled plasma samples obtained from normal mice (10 μ L) and incubated for 60 min at 38 °C. These samples were filtered (as described below) before HPLC-UV analysis.

Preparation of the calibration curve

Standard UA (0, 1.0, 2.0, and 4.0 mg/dL) and Xan (0, 0.5, 1.0, and 2.0 mg/dL) solutions were prepared using reagent-grade chemicals at the time of use. Each (3 μ L) was subjected to HPLC

using the aforementioned conditions. The automatic peak area counts (C-R-8A) for each peak of interest were plotted against concentration (mg/dL) to generate a calibration curve.

Mouse model of hyperuricemia

Animal use was approved by the Committee for the Ethical Use of Experimental Animals of Setsunan University. The experimental protocol conformed to the Animal Experiment Guidelines of Setsunan University, which were modified from the guidelines of the Japanese Society for Pharmacology. All efforts were made to minimize animal suffering, reduce the number of animals used, and utilize alternatives to techniques. The animals were treated and maintained in a room maintained at $23 \pm 1^\circ\text{C}$ with 47-67 % humidity, under a 12 h light-dark cycle with lights on at 7:00 AM. To generate the mouse model of hyperuricemia, 8 week-old male ICR mice (Japan SLC, Shizuoka, Japan) were intraperitoneally (i.p.) administered potassium oxonate (250 mg/kg)^{7,9)}. After 1h, mice were orally administered 50 mg/kg (1.5 mg/mouse) allopurinol. EDTA-treated blood was collected from the tail vein at 1, 2, or 3 h after treatment. Plasma was prepared immediately and stored at -80°C until further analysis. Potassium oxonate, allopurinol, and plasma samples were suspended or dissolved in 0.5 % carboxymethylcellulose solution before administration to mice.

Preparation of plasma

We performed deproteinization using the chloroform-methanol method. Ten microliters of plasma was placed in the sample reservoir of a Cosmospin Filter G spin filter (pore size; $0.2\ \mu\text{m}$, Nacalai Tesque, Kyoto, Japan) with $100\ \mu\text{L}$ of methanol, $100\ \mu\text{L}$ of chloroform, and $90\ \mu\text{L}$ of

H_2O , and sonicated. The filters were centrifuged at 10,000 rpm for 60 min to collect the aggregated protein.

The filtrate was lyophilized (MicroVac MV-100; Tomy Seiko, Tokyo, Japan) for 90 min. Dried residue was dissolved in $15\ \mu\text{L}$ H_2O . The solution was injected ($3\ \mu\text{L}$) into the HPLC instrument and analyzed in triplicate, immediately.

Effect of hemolysis on HPLC for uric acid

Approximately 1 mL of blood was centrifuged and the plasma collected. The residual red blood cells (RBCs) (*ca.* $500\ \mu\text{L}$) were washed three times with 1mL of phosphate-buffered saline containing EDTA. To obtain an erythrocyte lysate, $100\ \mu\text{L}$ of the RBCs were hemolyzed using $200\ \mu\text{L}$ of deionized water. Plasma was mixed with the erythrocyte lysate to investigate the effects of hemolysis on the uric acid analysis. The samples tested were comparable to clinical hemolyzed samples: a, $10\ \mu\text{L}$ plasma; b, $7.5\ \mu\text{L}$ plasma and $2.5\ \mu\text{L}$ RBC solution; c, $5\ \mu\text{L}$ plasma and $5\ \mu\text{L}$ RBC solution; d, $4\ \mu\text{L}$ plasma and $6\ \mu\text{L}$ RBC solution; and e, $10\ \mu\text{L}$ RBC solution¹⁰⁾.

Plasma (sample a) and erythrocyte lysate mixtures (samples c and e) were diluted (1/1000) with physiological saline, and the UV-VIS (200–700 nm) spectra were measured. Additional UA solution for a recovery test was obtained from the standard 10 mg/dL UA solution, diluted sevenfold with distilled water. The concentration of the UA solution was calibrated using the quantitative value of standard solution ($n=10$, $\text{CV}=0.98\%$). Ten microliters each of samples a-e and the UA solution ($10\ \mu\text{L}$) were placed in the sample reservoir of a Cosmospin Filter G spin filter with $100\ \mu\text{L}$ of methanol, $100\ \mu\text{L}$ of chloroform, and $80\ \mu\text{L}$ of H_2O , and sonicated. Each sample was analyzed using our HPLC method in triplicate.

Statistical analysis

The HPLC assays were performed in triplicate and the averages were calculated. All animal experiments used 7-10 mice, and the data are presented as means $\pm$ standard deviations¹¹⁾. Differences between the means of the control and sample-treated mice were analyzed using one-way analysis of variance and Student's *t*-test

Figure 1.
HPLC chromatogram on a used column (850 individual plasma analyses) of authentic uric acid and related compounds.

Common condition: The following compounds were detected by HPLC at 280 nm: Peak 1, hypoxanthine (0.8 mg/dL, t_R 5.6 min); Peak 2, uric acid (1.7 mg/dL, t_R 7.3 min); Peak 3, xanthine (0.6 mg/dL, t_R 9.1 min); Peak 4, oxipurinol (1.6 mg/dL, t_R 11.9 min); Peak 5, allopurinol (0.7 mg/dL, t_R 13.5 min). Cosmosil 5C18-AR-II 4.6 $\times$ 250 mm column: injection volume 3 μ L, temperature 30 $^{\circ}$ C, mobile phase 30 mmol/L aqueous phosphoric acid solution, range 0.02, attenuation 1. R_s value (peaks 2 and 3) was 4.33, for the four-compound (2-5) mixture separated on the same new column (data not shown). The R_s value (peaks 2 and 3) was 2.01 for a five-compound mixture separated on one column used for 1200 individual plasma analyses (data not shown).

with Bonferroni's correction. A *P*-value of <0.05 was deemed to indicate statistical significance.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

A representative chromatogram of authentic oxypurines is shown in Figure 1.

Isocratic separation of a standard mixture of five compounds was achieved within 15 min with adequate reproducibility, as indicated by a coefficient of variation of 0.98 %. Therefore, we successfully determined the optimum conditions for quantitative analysis of plasma UA levels using an improved HPLC-UV method.

The determined HPLC conditions showed stable high separation performance and column durability. Briefly, a mixed test solution (four or five compounds) was prepared using standard solutions. The suitability of the stationary (column) and mobile phases was evaluated using the mixed standard solution. Five types of ODS column (4.6 $\times$ 250 mm, carbon content 10–17 %, particle size 3–15 μ m) were compared. The performance of each of these in combination with mobile phases consisting of an aqueous trifluoroacetic acid solution (100 μ L/L, pH 4.0) or aqueous phosphoric acid solution (20 mM, pH 3.5) at a flow rate of 1.0 mL/min were compared. Of the ten combinations, the chromatograms indicated that Cosmosil 5C18-AR-II separation in aqueous phosphoric acid solution was optimum (no overlapping peaks). As a result, high separation (R_s and theoretical plates) of the five peaks was maintained. The phosphoric acid concentration in the mobile phase was such that elution was completed within 15 min; the durability of the column was also taken into consideration; thus the final phosphoric acid concentration used was 30 mM. Column temperature was maintained at 5 $^{\circ}$ C above room temperature for the purpose of baseline stability

(for separation performance only, a lower temperature results in superior separation). In addition, a reservoir was used to maintain the mobile phase at a fixed room temperature.

Further, the sample injection port and syringe were washed meticulously with purified water. Using these conditions, the UA peak on the chromatogram with a new column gave a theoretical plate value of 5100 and an R_s (column separation value, peaks 2 and 3) value of 4.33. The performance durability of the column was determined by analyzing 850 and 1200 plasma samples, which gave R_s =2.78 and 2.01, respectively.

The separation of uric acid and its derivatives with an ODS column depends on the end-cap reagent and bridge structure. The ODS column that we used can successfully separate and evaluate uric acid, xanthine, and other plasma contents.

Stability of uric acid in aqueous solution

The stability test examined the temporal change in uric acid dilution at 0, 1, 4, 72, 96, 120, and 168 h (Fig. 2).

The concentration of uric acid was linear from

Figure 2.

Stability of uric acid in aqueous solution.

Authentic samples (AP, Hyp, OP, Xan, and UA at 0.7, 0.8, 1.6, 0.6, and 1.7 mg/dL, respectively) were dissolved in deionized water, preserved in an air-tight container, and stored at room temperature (8-20 °C) for the indicated times. Each condition was determined in triplicate and the results are presented as the means $\pm$ S.D.

0 to 72 h (0-4 h, $r=1.0$ and 0-72 h, $r=0.95$). After 96h, the peak of the decomposition product ($\lambda_{\max}$ 260 nm) of uric acid was detected at 8.3 min (the other compounds were stable, and their chromatograms are not shown).

Identification of the peak of UA in the chromatogram

To identify the UA peak by HPLC, samples were subjected to enzymatic treatment (Fig. 3A-C). Uricase treatment diminished the UA

Figure 3.

Identification of uric acid in plasma by HPLC.

The following plasma samples were detected by HPLC at 280 nm. Three groups ($n=2$ per group) were evaluated under the same conditions:

- (A) Pooled plasma from control, non-treated mice.
- (B) Pooled plasma samples treated with xanthine oxidase.
- (C) Pooled plasma samples treated with xanthine oxidase and UA oxidase.

Peak*, hypoxanthine and unknown compounds overlapped.

Common conditions: Cosmosil 5C18-AR-II 4.6 $\times$ 250 mm column, injection volume 3 μ L, temperature 30 °C, mobile phase 30 mmol/L aqueous phosphoric acid solution, range 0.02, attenuation 1.

peak while xanthine oxidase did not, confirming that the peak can be attributed to UA. No unknown peaks were revealed in the region in which the UA peak was absent, confirming that the latter does not overlap with that of any other compounds and that the peak area represents the quantity of UA. Using the same conditions, it is possible to simultaneously determine UA, Xan, AP, and OP, but not Hyp, levels in plasma samples.

Therefore, our findings indicate that the high-sensitivity HPLC-UV method provided adequate quantification with little variation. The most suitable HPLC-conditions for oxypurines involved a combination of a polymeric high-carbonized ODS column and aqueous phosphoric acid solution. The durability of the column was confirmed by our findings, indicating the effectiveness of chloroform-methyl alcohol for deproteinization of plasma. Avoidance of the use of acid during pre-processing resulted in no

decline in precision due to column destabilization by remnant acid. This improved method can be adapted to the general screening of plasma uric acid levels.

Precision, detection limit, and quantification limit

The peak areas ($n=4$) of UA (0, 1.0, 2.0, and 4.0 mg/dL), and xanthine (0, 0.5, 1.0, and 2.0 mg/dL) were fitted to a calibration curve ($r=0.9999$ and 0.9964 , $S_R=1.002$ and 0.9474 , respectively, Fig. 4)¹¹. The measurable upper limit (range) is 7.2 mg/dl, calculated because of the solubility of uric acid in water. This assay ($n=10$) is reproducible, with a relative standard deviation (σ), a detection limit (3σ) of 0.042 mg/dL, a determination limit (10σ) of 0.14 mg/dL, and a coefficient of closing variation (σ/mean) of 0.98 %⁴), versus more than 2.7–6.8 % using standard methods¹).

Spiking experiments

Authentic hypoxanthine could be separated using HPLC (Fig. 1). However, as the peak of hypoxanthine overlapped the peak of an unknown substrate in plasma (Fig. 3), the following quantification focused on uric acid and xanthine. The recovery of UA added to 10 μ L pooled plasma samples from normal mice was $102.9 \pm 13.2\%$ (adding 2 mg/dL, $n=4$, data not shown) and $97.8 \pm 16.6\%$ (adding 1.4 mg/dL, $n=6$, Fig. 5C). This is consistent with Kuroiwa's method^{5,6}), and confirms that the quantity, reproducibility, and stability of UA remain unchanged.

The effects of hemolysis on our method of uric acid analysis were investigated by adding an erythrocyte lysate to the samples (a-e). Three micro liter of the RBC solution were subject to HPLC ($n=6$). The chromatogram shows that no peak was detected in the range 7.1–8.8 min, and no uric acid peak was detected

Figure 4.

Linearity of HPLC chromatogram of UA and Xan

The areas of the peaks in the chromatogram were compared with the amount of UA or Xan injected. Each dose was determined using four or five independent samples and each result is shown as a scatter plot. The determination coefficient for UA was 0.9999 and that for Xan was 0.9964.

Figure 5. Effect of hemolysis on HPLC.

(A) HPLC chromatogram of a deproteinized red blood cell lysate (sample e).

The elution of HPLC was evaluated using the absorbance at 280 nm. No peak was detected nor did a peak interfere where the peak of UA should be observed.

(B) UV-VIS spectrum of the model samples of hemolysis (a, c, and e).

Each sample was diluted 1,000 fold with physiological saline. The absorbance at 410 nm is due to hemoglobin. This indicates that the model samples are comparable to clinical hemolyzed samples.

(C) Recovery rate of UA in the presence of erythrocyte lysate.

A 1.4 mg/dL UA solution was mixed with the hemolyzed models, deproteinized, lyophilized, and determined by HPLC. **, Significant compared with the 0 % sample ($p < 0.01$).

The hemolysis model samples.

The erythrocyte lysate was diluted with normal plasma to generate a model of hemolyzed plasma (erythrocyte solution v/v %, a, 0 %; b, 25 %; c, 50 %; d, 60 %; and e, 100 %).

(Fig. 5A). The lysate did not change the HPLC chromatogram, and did not interfere with the isolation of uric acid. Although a peak due to the lysate was observed in the chromatogram at 280 nm, it could be distinguished from the uric acid peak and did not affect the isolation of uric acid.

In the range 300–700 nm (Fig. 5B), the plasma (a) resulted in no absorption, and the erythrocyte lysate (c and e) showed the absorption due to hemoglobin (410 nm) and another absorption band (*ca.* 500–600 nm). An unknown amount of hemolysis in samples will prevent the accurate measurement of UA levels using an automated analyzer (detected 4-aminoantipyrine derivatives at 555 nm). In our method, hemolysis tends to lower the detection level of uric acid, although

slight hemolysis in blood samples will not affect the results (Fig. 5C).

Application to murine plasma samples

Hyperuricemia was induced by administration of potassium oxonate; plasma UA levels were then determined (Fig. 6). The UA level in the plasma oxonate-administered mice was significantly elevated compared to that of normal mice¹², and was significantly lowered by administration of allopurinol. Since our HPLC-UV method requires only a small volume of plasma, time-course studies using live mice are feasible (Fig. 6).

CONCLUSION

Uric acid and other oxypurines were assayed by HPLC-UV using a polymeric-ODS with a

Figure 6. Application of the HPLC method to a mouse model of hyperuricemia

Mice were administered oxonate to induce hyperuricemia, and given allopurinol or solvent orally. Blood was collected and plasma uric acid levels were determined by HPLC. The results are presented as means $\pm$ SD ($n=9$ or 10 per group).

(A) Time-dependent changes in the plasma uric acid levels. Squares represent control mice with normal levels; open diamonds, oxonate-treated hyperuricemic mice; circles, oxonate and allopurinol-treated mice.

(B) The data at 3 h in (A) were extracted. The statistical significance of differences compared to the normal control ($\#$ $p<0.01$) and hyperuricemic ($*p<0.05$; $**p<0.01$) mice was determined.

high-carbon content (*ca.* 17 %) column. And phosphoric acid aqueous solution (pH 1.8) was used as the mobile phase. They showed a high degree of separation (R_s value of 4.33) and full column perseverance in the chromatograms. The HPLC method is more sensitive (detection limit of 0.04 mg/dL) than the Enzyme-UV (VIS) method and hemolysis ($\sim$ *ca.* 30 %) causes less interference¹³⁾. The results confirmed that the preparation of the hyperuricemia model in this experiment and the measurement of the plasma uric acid levels in mice were being done properly. Moreover, the HPLC method needs less plasma, which means the diagnosis can be made with less invasive testing.

[Conflict of Interest (COI) : Nopotential COI to disclose]

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