Research article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 16 December 2015, Revised 29 January 2016, Accepted 22 February 2016 Published online 22 March 2016 in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.3390

A highly sensitive assay for xanthine oxidoreductase activity using a combination of [$^{13}C_2$,$^{15}N_2$]xanthine and liquid chromatography/triple quadrupole mass spectrometry

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In this study, we developed a highly sensitive assay for xanthine oxidoreductase (XOR) activity utilizing a combination of [$^{13}C_2$,$^{15}N_2$]xanthine and liquid chromatography (LC)/triple quadrupole mass spectrometry (TQMS). In this assay, the amount of [$^{13}C_2$,$^{15}N_2$]uric acid (UA) produced by XOR was determined by using LC/TQMS. For this assay, we synthesized [$^{13}C_2$,$^{15}N_2$]xanthine as a substrate, [$^{13}C_2$,$^{15}N_2$]UA as an analytical standard, and [$^{13}C_3$,$^{15}N_3$]UA as an internal standard. The [$^{13}C_2$,$^{15}N_2$]UA calibration curve obtained using LC/TQMS under the selected reaction monitoring mode was evaluated, and the results indicated good linearity ($R^2 = 0.998$, weighting of $1/x^2$) in the range of 20 to 4000 nM. As a model reaction of less active samples, the XOR activity of serial-diluted mouse plasma was measured. Thereby, the XOR activity of the 1024-fold-diluted mouse plasma was 4.49 ± 0.44 pmol/100 μL/h (mean ± standard deviation, $n = 3$). This value is comparable to the predicted XOR activity value of healthy human plasma. Hence, this combination method may be used to obtain high-sensitivity measurements required for XOR activity analysis on various organs or human plasma.

Keywords: stable isotope-labeled substrate; xanthine oxidoreductase activity; LC/TQMS; xanthine; uric acid

Introduction

Xanthine oxidoreductase (XOR) is a ubiquitous enzyme catalyzing the oxidation of hypoxanthine to xanthine and xanthine to uric acid (UA) in the purine degradation pathway (Figure 1a). In mammals, XOR exists in two convertible forms; xanthine oxidase (XO, EC 1.1.3.22), one form of XOR, generates oxygen-free radicals. Recently, it is widely reported that the elevated XOR activity was associated with various pathologies such as obesity,[1] type-2 diabetes,[2] coronary artery disease,[3] hypertension,[4] or final stage renal disease.[4] Studies of pathological mechanisms require accurate and sensitive measurements of XOR activity. We therefore developed the novel XOR activity assay with a combination of a stable isotope-labeled [$^{15}N_2$]xanthine and liquid chromatography (LC)/high-resolution mass spectrometry (HRMS). In the study, we use a [$^{15}N_2$]xanthine as substrate, and determine the amount of [$^{15}N_2$]UA, which is produced by XOR using LC/HRMS (Figure 1b). We reported the results of the XOR activity of mouse liver, kidney, and plasma.[5] However, this method is not sensitive enough to provide accurate measurements of the XOR activity of small tissues such as glomus, renal tubules, retina, or neuron. In addition, clinical studies also require a higher sensitivity assay because human plasma XOR activity is lower than that of mouse.[6]

In the present study, we have improved the sensitivity of the XOR activity assay based on our previous methods[5] by using TQMS instead of HRMS, because the sensitivity of TQMS is higher than that of HRMS. However, we estimated that the method utilizing [$^{15}N_2$]xanthine as the substrate was insufficient for TQMS because of its low resolution. To address this issue, we decided to use a [$^{13}C_2$,$^{15}N_2$]xanthine as the substrate and to

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Abbreviations: *XOR, xanthine oxidoreductase; LC, liquid chromatography; HRMS, high-resolution mass spectrometry; TQMS, triple quadrupole mass spectrometry; ESI, electrospray ionization; SRM, selected reaction monitoring; UA, uric acid; XDH, xanthine dehydrogenase; XO, xanthine oxidase; ISTD, internal standard; LLOQ, lower limit of quantification.*

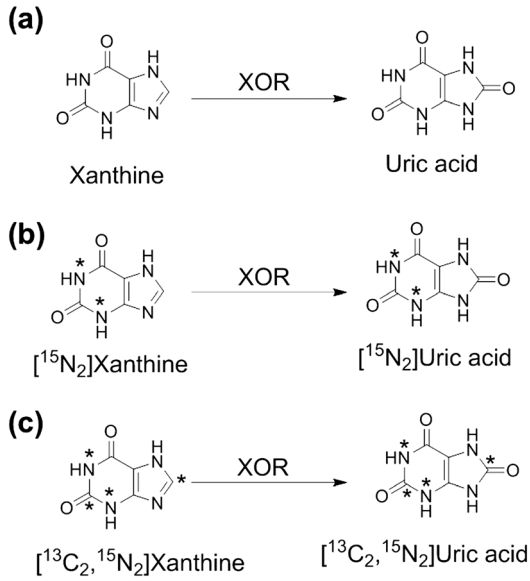


Figure 1. (a) Non-labeled xanthine oxidation by xanthine oxidoreductase (XOR) (b) [$^{15}N_2$]Xanthine oxidation by XOR (c) [$^{13}C_2$,$^{15}N_2$]Xanthine oxidation by XOR.

determine the amount of [$^{13}C_2$,$^{15}N_2$]UA produced by XOR using LC/TQMS (Figure 1c). Hence, we synthesized [$^{13}C_2$,$^{15}N_2$]xanthine as the substrate, [$^{13}C_2$,$^{15}N_2$]UA as the analytical standard, and [$^{13}C_3$,$^{15}N_3$]UA as its internal standard (ISTD). We now report that these synthesized compounds are suitable for the highly sensitivity XOR activity assay.

Experimental

Chemicals, reagents, and materials

[^{13}C,$^{15}N_2$]Urea, [^{13}C]urea, potassium [^{13}C]cyanide, and sodium [$^{15}N_2$]nitrite were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, U.S.). Ethyl acetamidocyanoacetate was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Methanol (MeOH, LC/MS grade) and ultrapure water (LC/MS grade) were obtained from Kanto Chemical (Tokyo, Japan). All other chemicals and reagents used came from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pooled normal human plasma was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Ultrafiltration Membrane Centrifugal Filter Plates (AcroPrep™ Advance 96-Well Filter Plates for Ultrafiltration, Omega™ 3 K MWCO) were purchased from Pall Corporation (Washington, NY, U.S.). The analytical column for LC/TQMS (Discovery HS F5, 150 mm × 2.1 mm i.d.) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). All other materials used were commercially available.

Methods

All new compounds were characterized by ^{1}H NMR (Varian 400-MR spectrometer, 400 MHz) with tetramethylsilane as the internal reference. High-resolution mass spectra were measured using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Low-resolution mass spectra were measured using an SQ Detector (Waters, Milford, MA, USA) equipped with an ESI source.

*[^{13}C,^{15}N]5,6-Diaminopyrimidine-2,4(1H,3H)-dione hemisulfate ([^{13}C,^{15}N]**2**)*

Method A: Sodium methoxide (1.23 g, 22.8 mmol) was added to a suspension of **1** (1.62 g, 9.53 mmol) and [^{13}C,$^{15}N_2$]urea (500 mg, 7.94 mmol) in tetrahydrofuran (THF; 14 mL) at room temperature. The mixture was heated, ethanol (EtOH) (4 mL) was added, and it was refluxed for 3 h. After cooling in an ice bath, a precipitate was filtered and dried at 45 °C *in vacuo* to give a powder (2.13 g). The powder was heated with 2 M sulfuric acid (H_2SO_4) (21 mL) and stirred for 10 min at 100 °C. After cooling, the precipitate was filtered, washed with water (2 mL), and dried at 45 °C *in vacuo* to give [^{13}C,^{15}N]**2** (635 mg, 41% yield) as a gray powder: ^{1}H NMR (DMSO-d_6) δ 6.10 (2H, s, NH_2), 7.85 (3H, brs, NH_2 and NH), 10.6 (1H, d, J = 91.6 Hz, NH); MS (ESI) *m/z* 146 $[M+H]^+$, 144 $[M-H]^-$.

Method B: Ethyl 2-cyanoacetate (905 mg, 8.01 mmol) was added dropwise to a mixture of [^{13}C,$^{15}N_2$]urea (500 mg, 7.93 mmol) and 20% sodium ethoxide in EtOH solution (8.0 mL) with an ice bath. The resulting suspension was slowly heated to the reflux temperature and left to stand at 75 °C for 5 h. After cooling, water (15 mL) was added and then acetic acid (AcOH) (1.5 g) to give a suspension. The suspension was stirred for 30 min in an ice bath, filtered, and dried *in vacuo* at 50 °C overnight to give [^{13}C,$^{15}N_2$]6-aminopyrimidine-2,4(1*H*,3*H*)-dione ([^{13}C,$^{15}N_2$]**4**) (0.867 g, 84% yield) as a pale gray powder: ^{1}H NMR (DMSO-d_6) δ 4.39 (1H, t, J = 2.8 Hz, C5-H), 6.30 (2H, s, NH_2), 10.0 (1H, d, J = 91.6 Hz, NH), 10.4 (1H, brs, NH); MS (ESI) *m/z* 131 $[M+H]^+$, 129 $[M-H]^-$.

Compound [^{13}C,$^{15}N_2$]**4** (0.861 g, 6.62 mmol) and sodium nitrite (502 mg, 7.28 mmol) were resolved in a 2 M sodium hydroxide (NaOH) aqueous solution (9.0 mL). The solution was added dropwise to frozen AcOH (11.1 mL) in an ice bath, and the purple suspension was stirred for 10 min in the ice bath. The precipitate was filtered, washed with water (1 mL × 3), and dried *in vacuo* at 40–50 °C overnight. The obtained purple solids (1.05 g) were suspended in water (15 mL) and heated to 100 °C. Sodium dithionite (1.87 g, 25.3 mmol) was added to the suspension by portions. After the purple color disappeared, the suspension was cooled and 2 M H_2SO_4 (6.6 mL, 13.2 mmol) and water were added to it. The suspension was stirred at room temperature overnight, and the precipitates were filtered, washed with water (2 mL × 3), and dried *in vacuo* at 45 °C to give [^{13}C,$^{15}N_2$] **2** (1.08 g, 84% yield) as a pale brown powder: ^{1}H NMR (DMSO-d_6) δ 6.09 (2H, s, NH_2), 7.8 (3H, brs, NH_2 and NH), 10.6 (1H, d, J = 91.3 Hz, NH); MS (ESI) *m/z* 146 $[M+H]^+$, 144 $[M-H]^-$.

[$^{13}C_2$,$^{15}N_2$]UA

The hemisulfate [^{13}C,$^{15}N_2$]**2** (578 mg, 2.98 mmol) was neutralized with 1 M NaOH (2.98 mL, 2.98 mmol) and sonicated at room temperature. The resulting free form was precipitated, filtered, and dried at 40 °C *in vacuo* to provide a pale gray powder (368 mg, 2.54 mmol): ^{1}H NMR (DMSO-d_6) δ 2.95 (2H, brs, NH_2), 5.54 (2H, s, NH_2), 9.89 (1H, brd, J = 89.8 Hz, NH), 10.3 (1H, dd, J_1 = 91.1 Hz, J_2 = 2.3 Hz, NH). The free form [^{13}C,$^{15}N_2$]**2** (368 mg, 2.54 mmol) and [^{13}C]urea (170 mg, 2.8 mmol) were mixed and pulverized in a mortar, and then stirred at 185 °C for 1 h. The resulting mixture was dissolved in water (5 mL) and 1 M NaOH aqueous solution (9 mL) at 90 °C and filtered. The filtrate was acidified with 1 M hydrochloric acid (HCl) (12 mL), and the resulting precipitate was collected and dried at 40 °C *in vacuo* to give [$^{13}C_2$,$^{15}N_2$]UA (296 mg, 58% yield in two steps) as a pale yellow powder: ^{1}H NMR (DMSO-d_6) δ 10.5 (1H, s, N7-H), 10.7 (1H, d, J = 88.4 Hz, NH), 11.6 (2H, brs, NH × 2); HRMS calculated for $[C_3^{13}C_2H_4N_2^{15}N_2O_3+H]^+$: *m/z* 173.0364; found: 173.0361 (−1.7 ppm).

[$^{13}C_2$,$^{15}N_2$]Xanthine

The hemisulfate [^{13}C,^{15}N$_2$]**2** (0.15 g, 0.773 mmol) was stirred with [^{13}C]formamide (0.25 g, 5.41 mmol) at 210 °C for 2 h. The resulting mixture was dissolved in a 1 M NaOH aqueous solution (2.3 mL) at 90 °C and filtered. The filtrate was acidified with 1 M HCl (4.6 mL) at 4 °C, and the resulting precipitate was collected and dried at 40 °C *in vacuo* to give [^{13}C$_2$,^{15}N$_2$]xanthine (0.089 g, 74% yield): ^{1}H NMR (DMSO-d$_6$) δ 7.93 (1H, d, *J* = 211 Hz, C8-H), 10.8 (1H, d, *J* = 90.1 Hz, NH), 11.5 (1H, d, *J* = 93.8 Hz), 13.3 (1H, s, N7-H); HRMS calculated for [$C_3^{13}C_2H_4N_2^{15}N_2O_2$ + H]$^+$: *m/z* 157.0415; found: 157.0412 (−1.9 ppm).

*Ethyl [^{13}C,^{15}N]2-acetamido-2-cyanoacetate ([^{13}C,^{15}N]**1**)*

Potassium [^{13}C]cyanide (1.00 g, 15.1 mmol) was added to a solution of ethyl chloroacetate (2.03 g, 16.6 mmol) and 18-crown-6 (200 mg, 0755 mmol) in acetonitrile (10 mL), in an ice bath. The mixture was stirred at room temperature for two days and filtered, and the solid washed using acetonitrile (10 mL). The filtrate was concentrated under 70 Torr at 30–35 °C to give crude [^{13}C]**3** (2.52 g) as an oil: ^{1}H NMR ($CDCl_3$) δ 1.32 (3H, t, *J* = 7.1 Hz, $CH_2\underline{CH_3}$), 3.49 (2H, d, *J* = 10.0 Hz, C2-CH_2), 4.27 (2H, q, *J* = 7.1 Hz, $\underline{CH_2}CH_3$).

Sodium [^{15}N]nitrite (1.00 g, 14.3 mmol) and, subsequently, a solution of AcOH (948 mg, 15.8 mmol) in water (0.55 mL) was added to the crude [^{13}C]**3** (2.52 g) in water (0.51 mL), in an ice bath. The mixture was stirred at room temperature overnight. After ethyl acetate (20 mL) and 5% sodium bicarbonate aqueous solution (15 mL) were added to the reaction mixture in the ice bath, the organic layer was separated, and the aqueous layer was extracted using ethyl acetate (20 mL × 2). The organic layers were combined, dried over sodium sulfate, concentrated, and dried *in vacuo* at 40 °C to give crude ethyl [^{13}C,^{15}N]2-cyano-2-hydroxyiminoacetate ([^{13}C,^{15}N]**6**) (1.46 g).

A suspension of the crude [^{13}C,^{15}N]**6** in AcOH (2.0 mL) was added slowly to a suspension of zinc powder (3.22 g) in a solvent of acetic anhydride (3.0 mL) and AcOH (3.0 mL), in an ice bath at 7–15 °C under nitrogen. Additional AcOH (1 mL × 2) was used to wash the vessel of the crude [^{13}C,^{15}N]**6**. The mixture was stirred overnight at room temperature. The resulting mixture was filtered, and the insolubles were washed with AcOH (4 mL × 2). Thereafter, the insolubles were suspended in a mixed solvent of AcOH (2 mL) and chloroform (5 mL), and filtered; this washing was repeated, and the filtrates were combined and concentrated. The residue was solved in chloroform (20 mL), and NaOH aqueous solution 5% (25 mL) was added at 5–10 °C. The organic layer was separated, and the aqueous layer was extracted with chloroform (30 mL × 2). The organic layers were combined, dried over anhydrous sodium sulfate, and concentrated to give a white solid (1.62 g). The solid was refluxed in ethyl acetate (5 mL) and left to stand first at 20 °C for 2 h, and subsequently, at 6 °C for three days. The obtained crystals were filtered, dried *in vacuo* at 40 °C for 3 h to give [^{13}C,^{15}N]**1** (755 mg) as colorless crystals. The filtrate was concentrated, and subjected to silica gel column chromatography (n-hexane:ethyl acetate = 2:1) to give [^{13}C,^{15}N]**1** (185 mg). The total amount of [^{13}C,^{15}N]**1** was 940 mg obtained in 36% yield from potassium [^{13}C]cyanide: ^{1}H NMR ($CDCl_3$) δ 1.37 (3H, t, *J* = 7.1 Hz, $CH_2\underline{CH_3}$), 2.12 (3H, d, *J* = 1.5 Hz, CH_3CO), 4.37 (2H, q, *J* = 7.1 Hz, $\underline{CH_2}CH_3$), 5.53 (1H, ddd, J_1 = 11.6 Hz, J_2 = 7.6 Hz, J_3 = 1.7 Hz, C2-H), 6.31 (1H, ddd, J_1 = 93.4 Hz, J_2 = 7.6 Hz, J_3 = 0.9 Hz, NH); MS (ESI) *m/z* 173 [M + H]$^+$, 171 [M − H]$^-$.

*[$^{13}C_2$,$^{15}N_3$]5,6-Diaminopyrimidine-2,4(1H,3H)-dione hemisulfate ([$^{13}C_2$,$^{15}N_3$]**2**)*

Sodium methoxide (781 mg, 14.5 mmol) was added to a suspension of [^{13}C,^{15}N]**1** (913 mg, 5.31 mmol) and [^{13}C,^{15}N$_2$]urea (304 mg, 4.83 mmol) in THF (9 mL), at room temperature. The mixture was heated, EtOH (2.5 mL) was added, and it was refluxed for 3 h. After cooling, the precipitate was filtered and dried at 45 °C *in vacuo* to give a powder (1.41 g). The powder was heated with 2 M H_2SO_4 (13 mL) and stirred for 10 min at 100 °C. After cooling in an ice bath, the resulting precipitate was filtered, washed with water, and dried at 45 °C *in vacuo* to give [^{13}C$_2$,^{15}N$_3$]**2** (172 mg, 18% yield): ^{1}H NMR (DMSO-d$_6$) δ 6.10 (2H, s, NH_2), 7.7 (3H, brs, NH_2 and NH), 10.64 (1H, d, *J* = 91.4 Hz, NH); MS (ESI) *m/z* 148 [M + H]$^+$, 146 [M − H]$^-$.

[$^{13}C_3$,$^{15}N_3$]UA

The hemisulfate [^{13}C$_2$,^{15}N$_3$]**2** (0.170 g, 0.867 mmol) was mixed with [^{13}C]urea (0.0580 g, 0.954 mmol), pulverized in a mortar, and stirred at 190 °C for 2 h. The resulting residue was dissolved in 1 M NaOH (2.6 mL) at 90 °C and filtered. The filtrate was acidified with 1 M HCl (5.3 mL) at 4 °C, and the resulting precipitate was collected and dried at 40 °C *in vacuo* to give [^{13}C$_3$,^{15}N$_3$]UA (0.12 g, 80% yield) as a pale gray powder: ^{1}H NMR (DMSO-d$_6$) δ 10.5 (1H, d, *J* = 98.8 Hz, N7-H), 10.7 (1H, d, *J* = 88.9 Hz, NH), 11.6 (2H, brs, NH × 2); HRMS calculated for [$C_2^{13}C_3H_4N^{15}N_3O_3$ + H]$^+$: *m/z* 175.0368; found: 175.0364 (−2.3 ppm).

Preparation of enzyme extracts from mouse plasma

All animal experiments were approved by the Committee on Animal Care of Sanwa Kagaku Kenkyusho Co., Ltd. Retired ICR mice obtained from Charles River Laboratories (Yokohama, Japan) were sacrificed under 2.5% isoflurane anesthesia. Blood was collected in heparinized tubes and immediately centrifuged at 2000 × *g* for 10 min at 4 °C to obtain plasma.

Calibration curve of [$^{13}C_2$, $^{15}N_2$]UA with human plasma

To prepare calibration standard samples, each 100-μL pooled human plasma sample spiked with 20–4000 nM [^{13}C$_2$,^{15}N$_2$]UA was mixed with 2 μM [^{13}C$_3$,^{15}N$_3$]UA as ISTD and the total volume was adjusted to 250 μL using a Tris-buffer (pH 8.5); 500 μL MeOH was added, and the mixture was centrifuged at 2000 × *g* for 15 min at 4 °C, the supernatant was evaporated and reconstituted with 150 μL Tris-buffer. Each solution was filtered through an ultrafiltration membrane, and the amount of [^{13}C$_2$,^{15}N$_2$]UA was measured using LC/TQMS. The calibration curve was constructed by plotting the peak area ratio of [^{13}C$_2$,^{15}N$_2$]UA (Analyte) to [^{13}C$_3$,^{15}N$_3$]UA (ISTD) versus the concentrations of [^{13}C$_2$,^{15}N$_2$]UA.

Confirmation of sensitivity: XOR activity assay of diluted mouse plasma

The XOR activity was measured with the serially diluted mouse plasma as a model reaction of a less active biological sample to confirm the sensitivity of the assay utilizing [^{13}C$_2$,^{15}N$_2$]xanthine. A 100-μL mouse plasma sample diluted 1, 4, 16, 64, 256, or 1024 times was mixed with 16 μM [^{13}C$_2$,^{15}N$_2$]xanthine, 40 μM NAD$^+$, 7.98 μM oxonate, and 2 μM [^{13}C$_3$,^{15}N$_3$]UA, and the total volume was adjusted

to 250 μL using a Tris-buffer (pH 8.5). After incubation at 37 °C for 90 min, 500 μL MeOH was added, and the mixture was centrifuged at 2000 × *g* for 15 min at 4 °C, the supernatant was evaporated and reconstituted with 150 μL Tris-buffer. Each solution was filtered through an ultrafiltration membrane, and the amount of [$^{13}C_2$,$^{15}N_2$]UA was measured using LC/TQMS.

LC/TQMS conditions and data analysis

The LC separation was performed using high-performance LC (Nano space SI-2, Shiseido, Tokyo, Japan). A 20-μL sample was loaded onto an analytical column at 40 °C. Mobile phase A was 0.5% AcOH/ultrapure water, and mobile phase B was 0.5% AcOH/MeOH. The analytical gradient profile was as follows (min/B%): 0/0, 5.0/2.5, 5.1/0, and 7.0/0. The flow rates were 200 μL/min. MS analysis was performed using a triple quadrupole mass spectrometer (TSQ Quantum, Thermo Fisher Scientific GmbH, Bremen, Germany) with a positive ESI selected reaction monitoring (SRM) mode. The SRM transitions (precursor ion → product ion) for the [$^{13}C_2$,$^{15}N_2$]UA and [$^{13}C_3$,$^{15}N_3$]UA were *m/z* 173 → 114 and 175 → 116, respectively. Peak area ratio value was obtained by dividing the peak area of [$^{13}C_2$,$^{15}N_2$]UA by that of [$^{13}C_3$,$^{15}N_3$]UA. XOR activities were expressed as [$^{13}C_2$,$^{15}N_2$]UA in pmol per hour.

Results and discussion

Effect of endogenous UA on XOR activity

Figure 2 presents the simulated mass spectrum of protonated non-labeled UA [$C_5H_4N_4O_3 + H^+$] calculated by Xcalibur 2.1 software (Thermo Scientific, MO, U.S.). There are several isotope ions besides the monoisotopic mass of *m/z* 169. As shown in Table 1, the relative abundance of the nominal mass of *m/z* 171 is 0.83%, although the calculated exact mass of *m/z* 171.0297 ($^{15}N_2$) is 0.0082%, because TQMS cannot distinguish [$^{15}N_2$]-isotope from other *m/z* 171 isotopes (e.g., [$^{13}C_2$] or [^{18}O]) because of its low resolution. The UA level in healthy human plasma is less than 7.0 mg/dL (absolute amount: 41.7 nmol/100 μL).[7] If 100 μL healthy human plasma is measured with TQMS, the *m/z* 171 peak delivered from endogenous UA will be detected at approximately 346 pmol equivalent of UA (41.7 × 1000 ÷ 100 × 0.83 = 346). On the other hand, according to the XOR activity results reported by Yamamoto

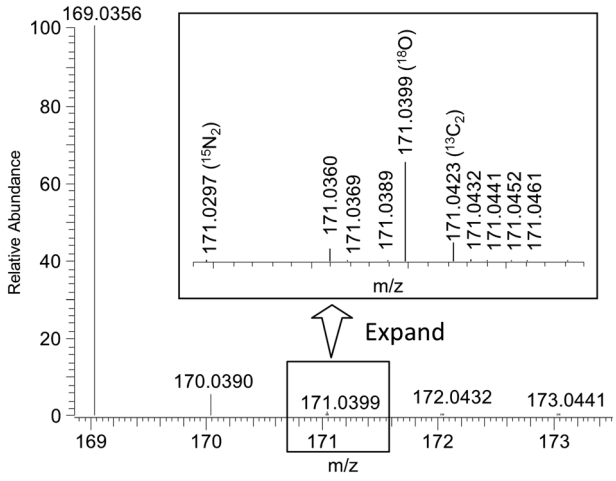


Figure 2. Simulated mass spectrum of protonated non-labeled uric acid [$C_5H_4N_4O_3 + H^+$] calculated by Xcalibur 2.1 software (Thermo Scientific., MO, U.S.).

Table 1. Relative abundance of nominal mass and calculated exact mass of monoisotope (*m/z* 169), a part of second-isotope (*m/z* 171), and a part of fourth-isotope (*m/z* 173) of uric acid

Nominal mass (TQMS)		Calculated exact mass (HRMS)		
m/z	Relative abundance	*m/z*		Relative abundance
169	100%	169.0356		100%
171	0.83%	171.0297	$^{15}N_2$	0.0082%
		.	.	.
		.	.	.
		.	.	.
		171.0399	^{18}O	0.62%
		171.0423	$^{13}C_2$	0.12%
		.	.	.
		.	.	.
		.	.	.
173	0.0026%			

et al. (healthy human plasma XOR activity measured with pterin as substrate)[8] and based on the results of our previous study (Xanthine oxidation rate by XOR is approx. 8 times higher than pterin oxidation rate by XOR),[5] we estimated that the plasma XOR activity of healthy human plasma measured with [$^{15}N_2$] xanthine will be approximately 10 pmol of [$^{15}N_2$]UA productions/100 μL/h, which is much lower than that of the *m/z* 171 peak delivered from endogenous UA (the former; 10 pmol, the later; 346 pmol). Thus, endogenous UA would greatly interfere

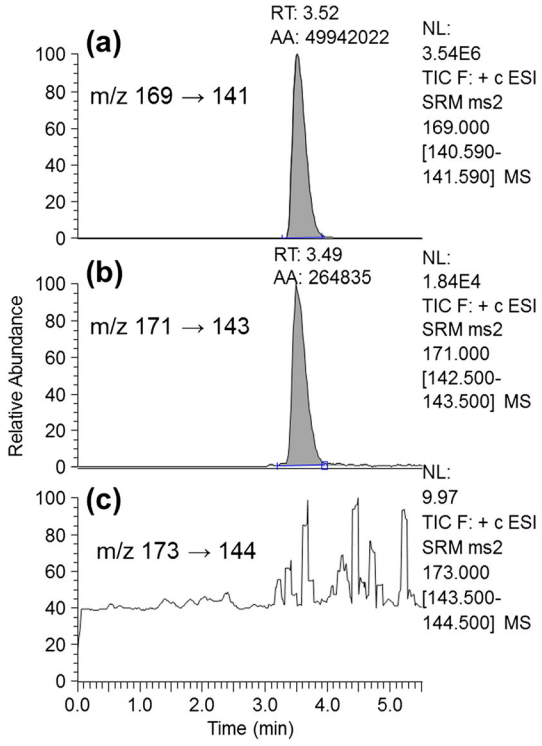


Figure 3. Selected reaction monitoring (SRM) chromatogram measured with liquid chromatography/triple quadrupole mass spectrometry. (a) Monoisotope of endogenous uric acid (SRM-transition; *m/z* 169 → 141), (b) second isotope (SRM-transition; *m/z* 171 → 143), and (c) fourth isotope (SRM-transition; *m/z* 173 → 144).

with [$^{15}N_2$]UA measuring in low-XOR activity samples, whereas it has a negligible effect on [$^{15}N_2$]UA measuring in high-XOR activity samples such as mouse tissue. Meanwhile, the relative abundance of nominal mass *m/z* 173 is 0.0026% (Table 1), which is 319 times lower than that of nominal mass *m/z* 171. This level corresponds to 10.8% of the estimated healthy human plasma XOR activity (346 ÷ 319 ÷ 10 × 100 = 10.8%), which has little influence on measuring XOR activity of human plasma. In fact, we measured the endogenous UA in 100 μL human plasma with TQMS. Figures 3a, 3b, and 3c present SRM chromatograms of protonated non-labeled UA monoisotope (SRM-transition; *m/z* 169 → 141), second-isotope (SRM-transition; *m/z* 171 → 143), and fourth-isotope (SRM-transition; *m/z* 173 → 144), respectively. Approximately 41.9 nmol endogenous UA was included in 100 μL human plasma. The second-isotopic peak (Figure 3b) was detected at 0.53% of the monoisotopic peak (Figure 3a), and the fourth-isotopic peak ((Figure 3c) was not detected. Based on these results, we decided to prepare [$^{13}C_2$,$^{15}N_2$] xanthine as substrate, [$^{13}C_2$,$^{15}N_2$]UA as an analytical standard, and [$^{13}C_3$,$^{15}N_3$]UA as an ISTD.

Syntheses of stable isotope-labeled compounds

The synthetic schemes are shown in Figures 4a–c. The common intermediate [^{13}C,$^{15}N_2$]**2** for labeled UA and xanthine was synthesized by the cyclization[9] of **1** and [^{13}C,$^{15}N_2$]urea with

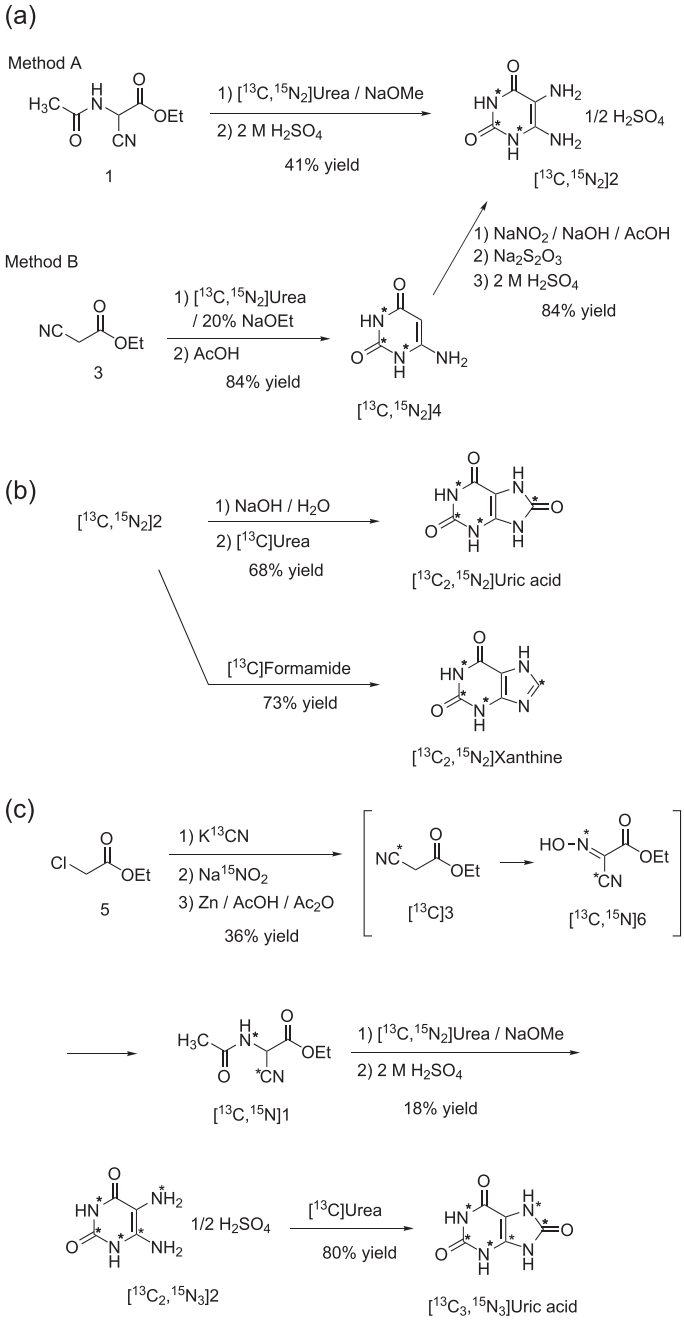
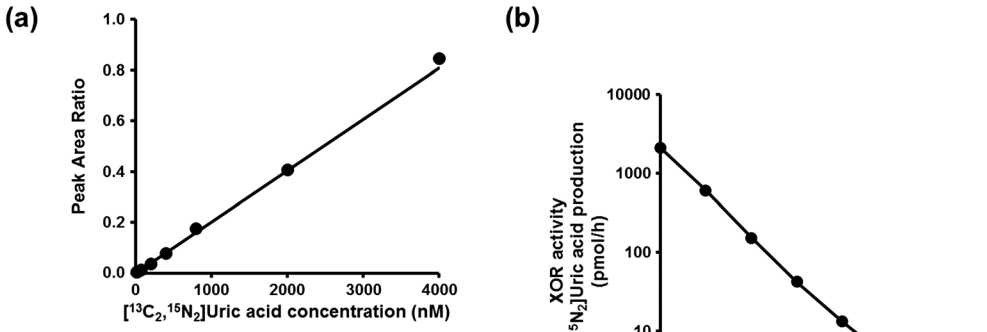


Figure 4. The synthetic schemes of [$^{13}C_2$,$^{15}N_2$]uric acid, [$^{13}C_2$,$^{15}N_2$]xanthine, and [$^{13}C_3$,$^{15}N_3$]uric acid. (a) Method A and Method B for the preparation of common intermediate [^{13}C,$^{15}N_2$]**2.** (b) Syntheses of [$^{13}C_2$,$^{15}N_2$]uric acid and [$^{13}C_2$,$^{15}N_2$]xanthine. (c) Synthesis of [$^{13}C_3$,$^{15}N_3$]uric acid.



Weighing factor, 1/x²

Nominal Concentration (nM)	4000	2000	800	400	200	80.0	40.0	20.0
Calculated Concentration (nM)	4170	2010	870	387	184	76.0	40.8	20.2
Accuracy (RE%)	4.3	0.4	8.8	-3.2	-8.2	-5.0	1.9	1.0

Figure 5. (a) Calibration curve of [$^{13}C_2$,$^{15}N_2$]uric acid using linear regression with a $1/x^2$-weighing factor for the determination of the [$^{13}C_2$,$^{15}N_2$]uric acid concentrations. (b) The XOR activity was measurement with the serially diluted mouse plasma as a model reaction of less-active biological samples. The horizontal axis represents the dilution ratio of mouse plasma, and the vertical axis represents XOR activity, which is expressed as produced pmol [$^{13}C_2$,$^{15}N_2$]uric acid per hour (mean ± standard deviation, $n = 3$).

sodium methoxide, followed by removal[10] of the acetyl group in 41% yield (Figure 4a, Method A). The obtained hemisulfate [$^{13}C_2$,$^{15}N_2$]**2** was transformed to a free form[11] and heated with [^{13}C]urea[12] to give [$^{13}C_2$,$^{15}N_2$]UA in 68% yield (Figure 4b). [^{13}C,$^{15}N_2$]Xanthine was formed from hemisulfate [^{13}C,$^{15}N_2$]**2** through heating with [^{13}C]formamide[13] in 73% yield. In this formation, the labeled formamide amount was decreased down to seven equivalents per mole hemisulfate from 48 equivalents, according to previous methods,[13] by using a sealed tube (Figure 4b). For the synthesis of [$^{13}C_3$,$^{15}N_3$]UA, potassium [^{13}C]cyanide and sodium [^{15}N]nitrite were used along with [^{13}C,$^{15}N_2$]urea and [^{13}C]urea. Ethyl 2-chloroacetate **5** was transformed to [^{13}C]**3** by substitution of potassium [^{13}C]cyanide,[14] and underwent an addition of sodium [^{15}N]nitrite[15] followed by reduction with zinc powder in 36% yield.[16] The obtained [^{13}C,^{15}N]**1** was cyclized to hemisulfate [$^{13}C_2$,$^{15}N_3$]**2** in 18% yield according to Method A in Figure 4a, and [$^{13}C_3$,$^{15}N_3$]UA was prepared from the hemisulfate in 80% (Figure 4c). In this preparation, the hemisulfate was heated with [^{13}C]urea without converting to the free form in order to increase the yield and prevent deep coloration. In addition, the synthetic method of [^{13}C,$^{15}N_2$]**2** was modified to acquire a steady yield as follows (Method B in Figure 4a). Ethyl 2-cyanoacetate was cyclized with [^{13}C,$^{15}N_2$]urea in a 20% sodium ethoxide EtOH solution to give [^{13}C,$^{15}N_2$]**4** in 84% yield.[17] A nitroso group was introduced at the fifth position of [^{13}C,$^{15}N_2$]**4** with sodium nitrate followed by reduction with sodium dithionite[18] to give [^{13}C,$^{15}N_2$]**2** in 84% yield. The accurate masses measured by HRMS of [$^{13}C_2$,$^{15}N_2$]xanthine, [$^{13}C_2$,$^{15}N_2$]UA, and [$^{13}C_3$,$^{15}N_3$]UA were within 3 ppm of the respective calculated masses.

Evaluating the standard calibration curve of [$^{13}C_2$,$^{15}N_2$]UA

We evaluated the standard calibration curve of [$^{13}C_2$,$^{15}N_2$]UA as the target analyte of the XOR activity assay. The result showed good linearity ($R^2 = 0.998$, weighting of $1/x^2$) between 20 and 4000 nM (Figure 5a). The LLOQ of [$^{13}C_2$,$^{15}N_2$]UA measured with LC/TQMS was 20 nM, whereas that of [$^{15}N_2$]UA measured with LC/HRMS is 667 nM.[5] Thus, detection sensitivity improved 33 times by the method developed in the present study.

Confirmation of sensitivity: XOR activity assay of diluted mouse plasma

As a model reaction of less-XOR active biological samples such as healthy human plasma, the XOR activity was measured with the serially diluted mouse plasma as an enzyme source (Figure 5b). The XOR activity of 1024-fold diluted mouse plasma was 4.49 ± 0.44 pmol per 100 μL per hour (mean ± standard deviation, $n = 3$), a value comparable to the predicted XOR activity value of healthy human plasma. Therefore, our developed method allows an accurate evaluation, even when the XOR activity is very low.

Conclusion

To develop a highly sensitive XOR activity assay, we synthesized [$^{13}C_2$,$^{15}N_2$]xanthine as the substrate, [$^{13}C_2$,$^{15}N_2$]UA as the analytical standard, and [$^{13}C_3$,$^{15}N_3$]UA as the ISTD. The LLOQ of [$^{13}C_2$,$^{15}N_2$]UA measured with TQMS was 20 nM, a value 33 times the corresponding HRMS-measured [$^{15}N_2$]UA value. Modeling less-active XOR samples, the XOR activity of 1024-fold-diluted mouse plasma samples was detected accurately. Consequently, the XOR activity assay utilizing a combination of [$^{13}C_2$,$^{15}N_2$] xanthine and TQMS is highly sensitive. In animal or clinical studies, this improved method may provide useful information regarding the participation of XOR in pathological conditions such as diabetic or renal disease.

Conflict of interest

The authors have declared no conflicts of interest.

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