Journal of Chromatography B, 1008 (2016) 189–197

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Xanthine oxidoreductase activity assay in tissues using stable isotope-labeled substrate and liquid chromatography high-resolution mass spectrometry

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ARTICLE INFO

Article history:
Received 17 April 2015
Received in revised form
16 November 2015
Accepted 17 November 2015
Available online 25 November 2015

Keywords:
High-resolution mass spectrometry
Xanthine oxidoreductase activity
Stable isotope-labeled substrate
Xanthine oxidoreductase inhibitor

ABSTRACT

Studies of pathological mechanisms and XOR inhibitor characterization, such as allopurinol, febuxostat, and topiroxostat, require accurate and sensitive measurements of XOR activity. However, the established assays have some disadvantages such as susceptibility to endogenous substances such as uric acid (UA), xanthine, or hypoxanthine. Here, we aimed to develop a novel XOR activity assay utilizing a combination of high-performance liquid chromatography (LC) and high-resolution mass spectrometry (HRMS) for tissues such as the liver, kidney, and plasma. Stable isotope-labeled [$^{15}N_2$]-xanthine was utilized as substrate and the production of [$^{15}N_2$]-uric acid was determined. [$^{15}N_2$]-UA production by XOR was dependent on the amounts of [$^{15}N_2$]-xanthine and enzyme and the time of reaction. Because high concentrations of endogenous xanthine and hypoxanthine affect XOR activities, we employed a multi-component analysis using LC/HRMS to improve the accuracy of XOR activity assay. Quantification of [$^{15}N_2$]-UA was validated and showed good linearity, accuracy, and precision. We measured the XOR activities of retired ICR mice using [$^{15}N_2$]-xanthine and LC/MS. The XOR activities in plasma, kidney, and liver samples were 38.1 ± 0.7, 158 ± 5, 928 ± 25 pmol/min/mg of protein, respectively (mean ± SD, $n = 5$). Furthermore, we measured the XOR activities in the same samples using the LC/ultraviolet and LC/fluorescence (FL) methods. The level of [$^{15}N_2$]-xanthine oxidation by XOR was equal to that of xanthine oxidation and approximately 7.9–8.9 times higher than that of pterin oxidation. We found a good correlation between XOR activities examined using LC/MS assay with [$^{15}N_2$]-xanthine and those examined using LC/FL assay with pterin. This result suggested that although both the LC/MS assay with [$^{15}N_2$]-xanthine and the LC/FL assay with pterin were useful, the former provided information regarding XOR activities that more directly reflected the physiological condition than the latter.

1. Introduction

Xanthine oxidoreductase (XOR) is an enzyme catalyzing the oxidation of hypoxanthine to xanthine and xanthine to uric acid (UA) in the purine degradation pathway. In the higher primates, including humans, UA is the ultimate metabolite in the pathway. In mammals, XOR exists in two convertible forms; one form is xanthine dehydrogenase (XDH, EC 1.1.1.204) and the other is xanthine oxidase (XO, EC 1.1.3.22). XDH reduces NAD^+ to NADH, whereas XO consumes oxygen to produce superoxide; hence, XOR activity implies the total activity of both forms [1]. In recent years, it has

Abbreviations: XOR, xanthine oxidoreductase; LC, liquid chromatography; HRMS, high-resolution mass spectrometry; UA, uric acid; UV, ultraviolet; FL, fluorescence; XDH, xanthine dehydrogenase; XO, xanthine oxidase; IXP, isoxanthopterin; ISTD, internal standard; LLOQ, lower limit of quantification; CV, coefficient of variation; RE, relative error; UPLC, ultra-performance liquid chromatography; TBS, Tris–buffered saline.

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http://dx.doi.org/10.1016/j.jchromb.2015.11.030
1570-0232/

been suggested that elevated XOR activity in plasma or several tissues (e.g., kidney, liver, heart, or adipose) is associated with aging [2–4], dialysis [5–7], or various pathologies such as obesity [8,9], type 1 diabetes [10], type 2 diabetes [11,12], and hypertension [7]. Moreover, XOR inhibitors such as topiroxostat (FYX-051) have been studied extensively [13]. Thus, it is important to measure the XOR activity accurately and sensitively for investigation in pathological studies and for the development of XOR inhibitors.

The enzymatic activity of XOR can be measured using several methods, including manometry [14], spectrophotometry [15], fluorometry [16–18], colorimetry [19], and radiometry [20,21]. XOR activity assay using xanthine as substrate and measuring the production of UA with ultraviolet (UV) detectors has been widely used in various studies [13,22,23]. Several studies use LC/UV to separate and quantify the product of the enzyme reaction UA [24,25]. In the assays using xanthine as substrate, the subtraction of endogenous UA measurement values from those resulting from the studied enzymatic reaction is necessary [25]. A recently widely used method is a fluorometric assay that utilizes pterin (2-amino-4-hydroxypteridine) as the substrate of XOR and measures isoxanthopterin (IXP) production in the presence of NAD^+ or methylene blue as an electron acceptor [16–18]. Although this assay is highly sensitive, pterin is not the main substrate for XOR in mammals, including humans; the affinity of pterin to XOR is lower than that of xanthine [16]. Radiochemical assays using [^{14}C]-xanthine [20] or [^{14}C]-hypoxanthine [21] are highly sensitive and the levels of endogenous UA present may be negligible. However, the method requires a radioisotope-dedicated facility and careful monitoring of radiation exposure. With the development of LC/MS technology, stable isotope-labeled compounds have been increasingly used in the studies of kinetics [26], metabolism [27,28], enzyme activity [29], and protein phosphorylation [30]. The advantages of LC/MS are that the stable isotope-labeled compounds may have the same physiological behavior as the native compounds, and these compounds are safer than the radioisotope-labeled compounds.

To address the issues associated with the conventional methods, we developed a novel XOR activity assay using [$^{15}N_2$]-xanthine as substrate, measuring [$^{15}N_2$]-UA production. We used liquid chromatography (LC) and high-resolution mass spectrometry (HRMS) and validated the results following the Food and Drug Administration bioanalytical guidelines [31].

2. Materials and methods

2.1. Standards, chemicals, and materials

[$^{15}N_2$]-UA, [$^{15}N_2$]-xanthine, [^{13}C]-urea, and [^{13}C,$^{15}N_2$]-urea were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, U.S.). Xanthine, hypoxanthine, NAD^+, and potassium oxonate were obtained from Sigma-Aldrich (St/Louis, MO, USA). Methanol (MeOH, LC/MS grade) and ultrapure water (LC/MS grade) were from Kanto Chemical (Tokyo, Japan). UA, methylene blue, pterin, and acetic acid (AcOH, LC/MS grade) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Isoxanthopterin (IXP) was purchased from Funakoshi (Tokyo, Japan). Protease inhibitor cocktail was purchased from Roche (Basel, Switzerland). BCA protein assays kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.). Sephadex G25 column (PD MiniTrap G-25) was from GE Healthcare (Little Chalfont, U.K.). Ultrafiltration Membrane Centrifugal Filter Units (Amicon® Ultra-0.5, 3K) were purchased from Merck Millipore (Billerica, MA, USA). Analytical columns for LC/MS (ACQUITY CSH fluoro-phenyl, 1.7-μm particle size, 100 mm × 1.0 mm i.d.) and for LC/fluorescence (FL) (ACQUITY HSS PFP, 1.8 μm particle size, 100 mm × 1.0 mm i.d.) were purchased from Waters (Milford, MA, USA). All other chemicals and materials used were commercially available.

2.2. Synthesis of [$^{13}C_2$,$^{15}N_2$]-UA for internal standard

An internal standard (ISTD), [$^{13}C_2$,$^{15}N_2$]-UA was synthesized in-house from [^{13}C]-urea and [^{13}C,$^{15}N_2$]-1,2-diaminouracil hemisulfate [32,33], which was synthesized from ethyl acetamidocyanoacetate with [^{13}C, $^{15}N_2$]-urea [34,35].

2.3. Preparation of enzyme extracts from mouse tissues

All animal experiments were approved by the Committee on Animal Care of Sanwa Kagaku Kenkyusho. Retired ICR mice, db/m mice and db/db mice, obtained from Charles River Laboratories (Yokohama, Japan) were fed standard laboratory chow. The mice were allowed ad libitum access to food and water and maintained in a plastic cage at a constant temperature of 23 ± 2 °C under a 12-h light–dark cycle (lights on from 7:00 am to 7:00 pm). After acclimatization for several weeks, the mice were sacrificed under 2.5% isoflurane anesthesia. Liver and kidney tissues were collected. Blood was withdrawn from the inferior vena cava of each mouse, mixed with heparin, and immediately centrifuged at 2000 × *g* for 10 min at 4 °C to collect plasma. The liver and kidney tissues were homogenized in 0.1 M phosphate buffered saline (pH 7.5) containing protease inhibitor cocktail, and they were centrifuged at 20,000 × *g* and 4 °C for 20 min. The supernatants were centrifuged at 105,000 × *g* and 4 °C for 60 min, and the cytosolic fraction was collected. The kidney cytosol was purified on a Sephadex G25 column. Protein concentration was determined using the BCA protein assay kit.

2.4. Optimization of enzymatic reaction conditions

2.4.1. The effect of [$^{15}N_2$]-xanthine amounts on [$^{15}N_2$]-UA production

The amounts of [$^{15}N_2$]-xanthine to be used in XOR activity assay were determined by examining the [$^{15}N_2$]-UA production at various concentrations of [$^{15}N_2$]-xanthine. On the basis of the results of preliminary examination (Supplementary figure), 7.5 μL liver cytosol, 30 μL kidney cytosol, and plasma of retired ICR mice were used in this section of the analysis. A 75 μL aliquot of 20 mM Tris–HCl, 150 mM NaCl buffer (Tris–buffered saline, TBS; pH 8.5) containing the cytosol/plasma was mixed with 12.5, 25, 50, 100, 200, 400, 800, or 1600 μM (presented as final concentration) of [$^{15}N_2$]-xanthine, 1 mM NAD^+, and 13.3 μM oxonate. The total volume was adjusted to 150 μL with TBS (pH 8.5). The mixtures were incubated at 37 °C for 30 min; 50 μL of 200 μM [$^{13}C_2$,$^{15}N_2$]-UA was added as ISTD. Subsequently, the mixture was heated for 5 min at 95 °C to stop the reaction. The resulting suspensions were centrifuged for 10 min at 15,000 × *g* and 4 °C. The supernatants were filtered through an ultrafiltration membrane, and the [$^{15}N_2$]-UA was quantitated using LC/MS.

2.4.2. The effects of reaction time and cytosol/plasma volume on [$^{15}N_2$]-UA production

Reaction time and the volume of the cytosol/plasma for XOR activity assay were determined by examining [$^{15}N_2$]-UA production for various volumes of cytosol/plasma and reaction times. TBS was added to different volumes of liver cytosol (2.5, 1.25, 0.625, or 0.313 μL), kidney cytosol (20, 10, 5.0, or 2.5 μL) or plasma (60, 30, 15, or 7.5 μL) to adjust the volume to 75 μL. These solutions were mixed with 400 μM [$^{15}N_2$]-xanthine (final concentration), 1 mM NAD^+, and 13.3 μM oxonate, and the total volumes were adjusted to 150 μL with TBS (pH 8.5). The mixtures were incubated at 37 °C for 15, 30, or 60 min. Then, the mixtures were treated, as described above, to obtain the samples for LC/MS analysis.