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A Sensitive and Specific Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination of Intracellular and Extracellular Uric Acid

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Abstract

Uric acid (UA) is known to be a major biological antioxidant in plasma. However, there is a strong correlation between UA levels and cardiovascular risk. Recent studies suggest that in the intracellular environment, UA can become a prooxidant that causes endothelial dysfunction. For conducting detailed studies of UA's role in human pathogenesis, there is a critical need for a sensitive and specific method for the determination of intracellular UA levels. We therefore developed a simple, sensitive method for determination of trace amounts of intracellular UA, as well as comparatively large amounts of UA in plasma and urine (for the determination of extracellular concentrations of UA), based on liquid chromatography and tandem mass spectrometry (LC-MS/MS). UA was separated from interferences by HPLC and quantified by mass spectrometry in the negative ESI mode using single reaction monitoring (SRM). For the identification and quantification of UA, the parent ions selected were m/z 167.0, which corresponds to the urate anion, and m/z 169.0, which corresponds to the 1,3-15N-UA anion. 1,3-15N-UA is used as an internal standard to ensure accuracy of the measurement. After precipitation of proteins with 10% TCA solution, UA was subjected to LC-MS/ MS analysis. The correlation coefficient was 0.9998 to 1.0000 based on the calibration curve. The intra- and inter-day precision (C.V. %) ranged from 0.01 to 3.07 and 0.01 to 3.68 in vivo and in vitro systems. Recovery tests of added standards have been successfully performed and the values ranged from 90.10 to 103.59 % and 98.74 to 106.12 % for in vivo and in vitro analyses, respectively. This study demonstrates that intracellular levels of UA can be measured using LC-MS/MS with isotope labeled UA as an internal standard.

Keywords

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1. Introduction

Uric acid (UA) is the end product of endogenous and dietary purine nucleotide metabolism in humans. Uric acid is generated by the xanthine oxidase—catalyzed conversion of xanthine and hypoxanthine [1]. Recently, UA has received attention not only because it forms crystals that are important in the pathogenesis of gout, but also because elevated plasma UA is associated with hypertension, insulin resistance and metabolic syndrome, and renal disease [2,3]. In particular, while UA is considered an antioxidant, recent studies suggest that soluble UA can also enter cells via specific transporters where it can induce proinflammatory and prooxidative effects. Thus, the intracellular UA concentration has been a key in the mediation of its cellular effects [4–7]. Therefore, the determination of the concentrations of intracellular and extracellular UA could lead to a better understanding of the clinical consequence of elevated UA as well assist in studies designed to test the biological effect of UA in cells.

Various methods for measuring uric acid have been studied in human body fluids. Enzymatic approaches for measuring UA, such as the uricase [8,9] and phosphotungstate [10] methods, have focused on concentrations in the plasma and urine and are limited in sensitivity as they can not detect levels less than 0.1mg/dl [11]. Assay of UA using enzymatic methods can also be imprecise, as the enzymatic method can be affected by the presence of metals; the non-enzymatic method can be affected by turbidity or the presence of aspirin, ascorbic acid, glutathione, paracetamol and various antibiotics [12]. Electrochemical methods have also been used to measure UA; however, ascorbic acid and dopamine present in biological samples can cause interference with these assays [13–15]. This led to the development of electrochemical methods that can selectively measure UA despite the presence of these substances (ascorbic acid and dopamine) in serum and urine [16–18].

An alternative approach is to measure UA levels using HPLC-UV [19–21] or less commonly GC-MS [22–24] as chromatographic methods. However, biological samples such as urine, plasma, serum, and tissue have much interference in the matrix so biological analyses require high sensitivity and selectivity. Unfortunately, HPLC-UV detection has low sensitivity and selectivity, so additional sample preparation is required for analyses [19–21]. To improve reproducibility, selectivity, and sensitivity, liquid chromatography-mass spectrometry (LC-MS) and LC-MS/MS analytical methods have been introduced as a means of determining biological substances. LC-MS can reduce analytical time and has the additional advantage of being able to measure nonvolatile and thermally labile compounds without any derivatization [25–28]. Perelló et al. published an LC-MS method for the determination of UA in various biological matrices but this method did not use an internal standard [29]. In another study, serum UA was measured using LC-isotope dilution mass spectrometry (ID-MS). Dai et al. applied 1,3-15N₂-UA as the isotopically labeled internal standard and suggested LC-IDMS method as an alternative reference method for analysis of serum uric acid [30]. We have recently developed an LC-MS/MS method and have used it to measure minute concentrations of UA within cells [31] and in plasma and urine. In this paper, we present the detailed methodology of this new approach. To further enhance accuracy, we used the commercially available isotope labeled UA (1,3-15N2-UA) as an internal standard. We document that we can accurately measure UA both intracellular and extracellular (plasma and urine) with excellent sensitivity, high specificity, high throughput, and a short run time. In particular, we believe that the use of this method is useful in the measurement of intracellular uric acid levels. This could be extremely important as there is increasing evidence that intracellular uric acid may have a role in hypertension, insulin resistance, and vascular disease.

2. Experimental

2.1. Chemicals

UA (purity >99%) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). $[1,3^{-15}N_2]$ -UA (purity >98%) was used as the internal standard (ISTD) and it was purchased from Cambridge Isotope Laboratories (Andover, MA, U.S.A). Ammonium acetate, acetic acid, potassium hydroxide (KOH), and methanol were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A). Trichloroacetic acid (TCA) was purchased from LabChem Inc (Pittsburgh, PA, U.S.A). HEPES buffer was obtained from Mediatech (Hernond, VA). The sample was filtered through 0.22 μ m Nylon centrifuge tube filter (COSTAR Corning Inc, NY, U.S.A). Fresh frozen human plasma was purchased from Civitan Regional Blood Center (Gainesville, FL, USA).

2.2. Preparation of standard solutions

Stock solution of UA was prepared at a concentration of 10 mM in 0.3 M KOH. The working solutions of various concentrations (0.1 and 1.0 mM) were prepared with 0.3 M KOH. All stock and working solutions were kept below 4°C. The ISTD was prepared by diluting a [1,3- $^{15}N_2$] UA stock solution at 1.0 mM with 0.3 M KOH. 10 % TCA was kept in the refrigerator before use.

2.3. Urine sample collection and preparation for UA analysis

Urine samples were stored at -80°C before analysis. For the analyses of urinary UA, urine samples were diluted 8-fold by volume with distilled water. The ISTD 1,3- $^{15}\text{N}_2$ -UA was added at a final concentration of 0.24 mM. 4 μL of internal standard and 200 μL 10 % TCA (w/v) were added to 1 mL diluted urine sample. This solution was vortexed for 30s and then filtered through a 0.22 μm Nylon filter and the filtrate was loaded into an autosampler vial and analyzed by LC-MS/MS.

2.4. Plasma sample collection and preparation for UA analysis

Plasma samples were stored at -80°C until analysis. In the case of UA analyses, each plasma sample (500 μ L), which included 0.24 mM of 1,3- $^{15}\text{N}_2$ -UA as the ISTD, was treated with 100 μ L 10 % TCA (w/v). The sample was vortexed and filtered through a 0.22 μ m filter by centrifugation at 14,000 rpm. The filtrate was loaded into an autosampler vial and analyzed by LC-MS/MS.

2.5. Cell culture and cell lysate preparation

Human umbilical vein endothelial cells (HUVECs, Clonetics) were plated on 60-mm culture plates and maintained at 37°C and 95% $O_2/5\%$ CO_2 in EBM BulletKit® Media (Clonetics). After cells grew at 90 % confluence, cells were then washed with PBS and trypsinized. The cell number was calculated by hemocytometer. Cells were lysed by with 0.3 M KOH, and then the lysate was sonicated. After the total volume, including total cell volume and volume of KOH solution, was calculated, the internal standard, $1,3^{-15}N_2$ -UA, was added to final concentration of 0.17 mg/dl. The samples were centrifuged at 136,000 rpm for 30 min and the supernatant was filtered through a 0.22 μ m Nylon filter at 3,000 rpm for 10 minutes. The filtrates were stored at -80° C for the measurement of UA by LC-MS/MS.

2.6. Instrumentation and LC-MS/MS conditions

The LC-MS analyses were carried out with a ThermoFinnigan Surveyor liquid chromatography system (ThermoFinnigan, San Jose, CA, USA) and a TSQ Quantum Discovery triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI interface operated in negative-ion mode detection. In the TSQ Quantum instrument, nitrogen was used for both the sheath and auxiliary gases at 60 and 20 arbitrary units, respectively. The

heated capillary temperature was maintained at 300° C. The collision pressure was 1.5 mTorr and the collision energy was 25V. The operation of the LC-MS and data analysis was performed using the ThermoFinnigan Xcalibur 1.4 software.

Liquid chromatography analyses were performed in a gradient elution mode using Phenomenex Luna 5μ C18(2) 100Å ($150\text{ mm} \times 4.6\text{ mm}$) column (Phenomenex, Torrance, CA, USA) coupled with a Phenomenex Luna C18 (2), 5μ particle size guard column. The mobile phase used included 5μ mM ammonium acetate/0.1 % acetic acid (A) and methanol (B). The mobile phase flow was $0.6\,\text{mL}\,\text{min}^{-1}$ and the flow was split (1:3) prior to the MS. The injection volume was $20\,\mu$ L. The gradient began at 95% A. The composition was linearly ramped to 25% B over the next $4.5\,\text{min}$, remained constant for $1.5\,\text{min}$, then reversed to the original composition of 95% A over $0.5\,\text{min}$, after which it was kept constant for $0.5\,\text{min}$ to re-equilibrate the column. UA was analyzed in the negative ESI mode and the parent ion of UA was m/z $167.0\,\text{and}$ monitored SRM ions were m/z $124.0\,\text{and}$ $96.0\,\text{L}$ in case of ISTD the parent ion of 1,3- 15N_2 -UA was m/z $169.0\,\text{and}$ monitored SRM ions were m/z $125.0\,\text{and}$ $97.0\,\text{L}$

2.7. Calibration

The quantification of UA *in vivo* and *in vitro* was carried out against a calibration curve prepared in each of the matrices. The UA standard concentrations were 1.0, 4.0, and 16.0 mg/dl for urine and plasma calibration curves. In the case of cell lysates, calibrators were applied at the concentrations of 0.0084, 0.017, 0.084, 1.000, and 4.000 mg/dl of UA. The ISTD $1.3^{-15}N_2$ UA was added at a final concentration of 4.0 mg/dl in urine and plasma and 0.17 mg/dl in cell lysate. The calibration curves were plotted as concentration vs. peak area ratio of the analytes and the ISTD.

2.8. Validation of the analytical method

Precision of the method was determined by calculating the intra-day and inter-day coefficients of variation (C.V. %) for UA. To measure the intra-day variance, five sets of prepared samples at each concentration level plus fixed concentration of internal standard were generated by using the urine and plasma or water stock solutions of the analytes. The intra-day variance was calculated based on five trial measurements accomplished in the first day, and the inter-day variance was calculated based on the results of five analyses carried out on five consecutive days. Accuracy of the method was determined by comparing measured concentrations with those added concentrations, and was expressed as RE (relative error, %). The calibration range for validation were as follows: 1) the range of 1.0 –16.0 mg/dl including 4.0 mg/dl of 1,3-15N₂-UA as ISTD in urine and plasma or water stock solutions, and 2) the range of 8.40 µg/dl - 4.00 mg/dl including 0.17 mg/dl of ISTD in cell lysate and water stock solutions. The recovery test for the protein precipitation was conducted using corresponding concentrations in water compared with samples spiked with UA in plasma, urine or cell lysates treated with 10 % TCA. For absolute values in each matrix, we first measured matrix samples of each matrix. The plasma and urine pools contained 4.46 mg/dl and 5.20 mg/dl of UA, respectively and these UA values were subtracted from the values of the matrix samples that had been spiked with isotope-labeled UA. This subtracted value was compared with the corresponding levels of UA in water samples.

2.9. Sample collection for normal plasma and urine

Fresh frozen human plasma (n=10) was purchased from Civitan Regional Blood Center (Gainesville, FL, USA). Subjects who provided urine samples included n=26 normal subjects from Baylor College of Medicine. Sample collection was approved by the local Institutional Review Boards for the Baylor College of Medicine and the University of Florida, respectively.

2.10. Measurement of intracellular UA levels in kidney proximal tubular cells treated with fructose

Human kidney proximal tubular cells (HK-2), an immortalized cell line from normal adult human kidney [32], were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown to confluence in Dulbecco's Modified Eagle Medium: Nutrient Mix (50/50) F-12 (DMEM/F-12) with L-glutamine and HEPES buffer (Mediatech, Hernond, VA). Cells were cultured at 37°C in 95% air and 5% carbon dioxide (CO₂) to 70–75% of confluence. Cells were harvested on ice in a buffer containing 25 mM HEPES (pH 7.1), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA and homogenized by 100 strokes of the pestle. 5 mM D-fructose was added to the cells to induce UA production and time intervals were 7 points (1, 5, 30, 120, 180, 1440, and 4320 min) after treatment of the cells with 5 mM D-fructose. The cells were rinsed with ice-cold PBS and harvested on ice in the buffer containing 25 mM HEPES (pH 7.1), 100 mM KCl, 1 mM DTT and 0.1 mM EDTA. The cell extracts were homogenized by 100 strokes of the pestle and frozen/thawed 4 times in liquid nitrogen. All samples were stored at -80°C prior to analysis. Before the analysis, they were thawed in a cold water bath and filtrated through a 0.2 µm Micro Centrifuge Nylon filter by centrifugation for 10min at 14,000 rpm. The filtrates were sealed in amber glass vials using Teflon-lined caps for LC-MS/MS analyses. The calibrators were 0.0084, 0.017, 0.084, 1.000, and 4.000 mg/dl UA.

3. Results

3.1. Typical total ion chromatograms (TIC) and internal standard

The SRM chromatograms of UA (panel A) and $1.3^{-15}N_2$ UA (panel B) under the experimental conditions are shown in Fig. 1. UA was measured in the negative ESI mode in urine, plasma, and cell lysates. MS/MS fragmentations using various collision energies were evaluated to determine the optimal MS/MS condition and they are shown in Fig. 2. The fragmentation pattern of UA was optimal in case of 25V (B panel) compared with other collision energies. As shown in panel B in Fig. 2, a major product ion of UA was m/z 124.0 ([M-NHCO]⁻) which was fragmented from a parent ion m/z 167.0 ([M-H]⁻) at a collision energy of 25V. The characteristic ion at m/z 96.0 was added for further confirmation of UA. $1.3^{-15}N_2$ -UA had fragmentation of m/z 125.0, 97.0, and 70.0 from m/z 169.0 in negative mode as shown in Fig. 2 (panel D).

3.2. Precision, accuracy and recovery

The linearity between ratio of peak area and the concentration of UA is shown in Table 1. The correlation coefficients and the equations are also presented in Table 1. The linear responses to UA were obtained for a range of 1.00 to 16.00 mg/dl in urine and plasma and from 8.40 μ g/dl to 4.00 mg/dl in cell lysates with correlation coefficients varying from 0.999 to 1.000. The recovery and intra- and inter-assay coefficients of variations were calculated by performing aliquots with water spiked with 1,3- 15 N₂ UA and each of the matrices (Table 1 and 2). The accuracy (% RE) varied between 0.03 to 7.93 % (intra-day) and 0.02 to 10.11 % (inter-day) and recoveries ranged between 90.10 to 106.12 % for *in vivo* and *in vitro* system. The precision (C.V. %) was 0.01 to 3.07 % for the intra-day and 0.01 to 3.68 % for inter-day assay for *in vivo* and *in vitro* system.

3.3. Determination of UA in normal samples

This new method has been used to determine UA concentrations in urine and plasma samples for the *in vivo* study. There was a high concentration of UA in human normal plasma (4.73 mg/dl, Fig. 3A) and urine (0.6 mg/dl, Fig. 3B) samples. In the case of HUVECs, UA was measured intracellularly using this method and these results are shown in Fig. 3C (0.05 mg/dl).

3.4. Time-dependent production of UA in cells treated with fructose

To examine whether elevated production of UA occurs in kidney tubular cells, we performed dose response and time course experiments with fructose loading. Time intervals were 7 points from 1 to 4320 min (72 hours) after treatment of the cells with 5 mM D-fructose. The value of UA was significantly increased about 7-fold at 5 min compared with 1 min after treatment with fructose and then UA concentration decreased gradually from 30 min. (Fig. 4).

4. Discussion

In this study, we developed and validated a method for the analysis of UA with stable isotope labeled UA as internal standard using LC-MS/MS in both the extracellular (plasma and urine) and intracellular (cell lysate) environment. The use of a stable isotope labeled UA as an internal standard allowed for increased accuracy. While Dia et al. recently published an LC-MS/MS method for the determination of UA in serum, we have developed a method that provides precise measurements of UA not only in plasma and urine samples, but also in the intracellular environment where UA concentrations were demonstrated to be in the μ g/dl range. Since increasing evidence suggests that the intracellular concentrations of UA may have profound cellular effects [4,33,34], this new methodology should be extremely helpful for studying the intracellular kinetics of UA...

The most common technique for measuring UA is the uricase method which was introduced in 1941 and uses a direct colorimetric procedure that measures serum and urine levels in the linear range between 0.4 to 25 mg/dl [35]. More sensitive techniques have utilized $^{15}\rm N$ isotopic methods for enrichment and quantitation of UA based on GC-MS [22–24]. Results using this method have been compared with those from an isotope dilution-gas chromatography-mass spectrometric method (ID-GC-MS), using $1,3^{-15}\rm N_2$ UA as internal standard and results in greater sensitivity, with detection of UA concentrations as low as 4.2 $\mu\rm g/dl$. Others have used reversed-phase liquid chromatography (LC) and HPLC with UV or amperometric detection to measure UA in various biological samples. In 1985, Zhiri et al., determined UA and creatinine in plasma simultaneously using an HPLC-UV detector. The assay was linear from 0.31 to 10.00 mg/dl and the limit of detection for UA was 0.16 mg/dl in plasma [36]. In another technique using electrochemical detection, Liu et al., measured UA, hypoxanthine and xanthine using HPLC with amperometric detection in plasma and was able to measure UA in the range 3.36 to 33.62 $\mu\rm g/dl$ [37].

In most studies, UA was measured in biological samples such as serum and urine. without [19–21,36,37] and with stable isotope labeled UA as the internal standard [22–24,30]. In our investigation, we used a stable isotope labeled 1,3- $^{15}N_2$ -UA that demonstrated an excellent linearity (correlation coefficients, r) of 0.999 to 1.000 with UA for plasma, urine, and cell lysates. The accuracy of quantification was internally controlled by comparing the peak area ratio between UA and 1,3- $^{15}N_2$ -UA versus mass concentration range. In cell lysates, the range of linear response was 8.40 µg/dl to 4.00 mg/dl and the limit of detection (LOD) of UA was 0.84 µg/dl. The LOD was calculated at a signal-to-noise ratio of 3. The limit of quantitation (LOQ) was 8.40 µg/dl at a signal-to-noise ratio of 169. The recovery values are shown in Table 1 and the recovery was better for the *in vivo* system than *in vitro* system. The reproducibility was good for both intra- and inter-day assays as shown in Table 2.

The typical range of blood UA is known to vary between 3.40 mg/dl and 7.00 mg/dl for males, and 2.40 mg/dl and 6.00 mg/dl for females [38–40]. The concentration of urinary UA is about 25.05 to 74.98 mg/dl in most healthy people [29]. In our study, the level of UA was 5.20 to 40.63 mg/dl in human urine and 4.29 to 9.09 mg/dl in human plasma. In the case of HUVECs, we calculated the normal range of intracellular UA to be 0.03 to 0.05 mg/dl. We also measured UA intracellularly in kidney tubular (HK2) cells in response to fructose treatment. In this case,

levels of intracellular UA were determined to be from 0.01 to 0.07 mg/dl. Fructose is known to generate UA within cells as a consequence of fructose-induced ATP depletion, intracellular phosphate reduction, and stimulation of AMP deaminase [41–43]. Our method was able to demonstrate that fructose could triple intracellular UA concentrations.

5. Conclusions

In this study, we have successfully developed a method using stable labeled UA as an internal standard for the analyses of UA using LC-MS/MS in human urine, plasma and cell lysates. The sensitivity and precision of this method allows accurate measurement of intracellular UA concentrations. We propose that this method could be useful to determine the relative role of intracellular and extracellular UA in various conditions such as cardiovascular disease, preeclampsia, and hypertension.

Acknowledgments

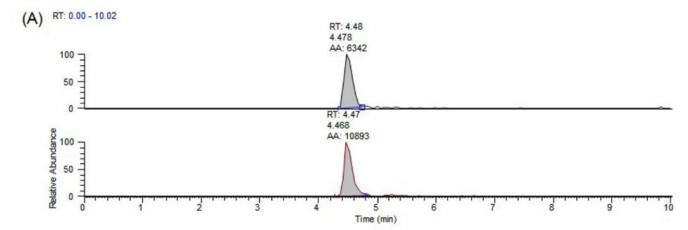
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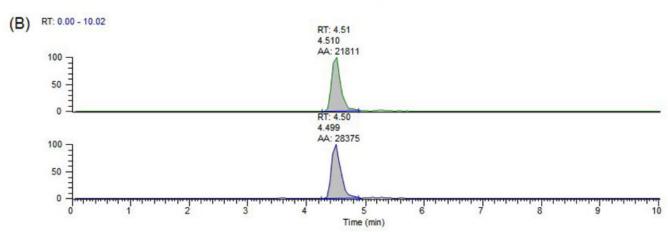


Figure 1. SRM chromatograms of UA and $1,3^{-15}N_2$ -UA standards. All of them were recorded at the collision energy of 25V and using ESI interface in the negative ion mode. (A); UA (1.00 mg/dl, up panel m/z 96.0: down panel is m/z 124.0), (B); $1,3^{-15}N_2$ -UA (4.51 mg/dl, up panel m/z 97.0: down panel is m/z 125.0)

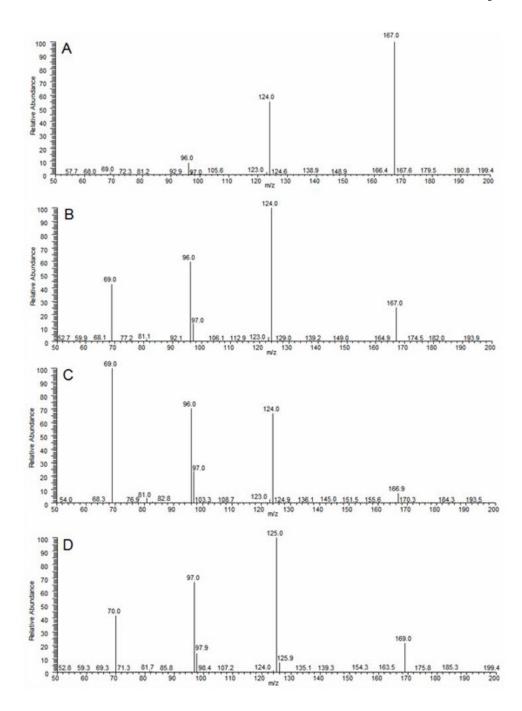


Figure 2. MS/MS spectra of UA and $1,3^{-15}N_2$ -UA standards determined using various collision energies. All of them were performed in the negative mode using ESI interface. The major fragmentation ions of UA were m/z 124.0, 96.0, and 69.0 from molecular ion m/z 167.0. A: collision energy 15V for UA, B; collision energy 25V for UA, C; collision energy 30V for UA, and D; collision energy 25V for $1,3^{-15}N_2$ -UA (fragmentation ions; m/z 125.0, 97.0, and 70.0 from molecular ion m/z 169.0)

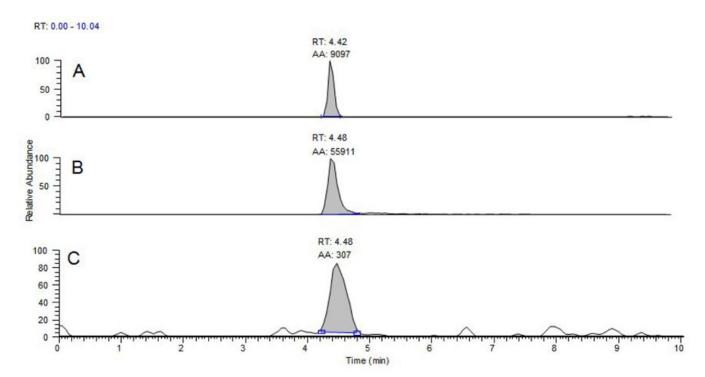


Figure 3. SRM TIC chromatograms of UA in normal plasma (A: 4.73 mg/dl), urine (B: 15.75 mg/dl), and HUVEC cell lysate (C: 0.05 mg/dl). All of them were applied the collision energy of 25V and used ESI interface.

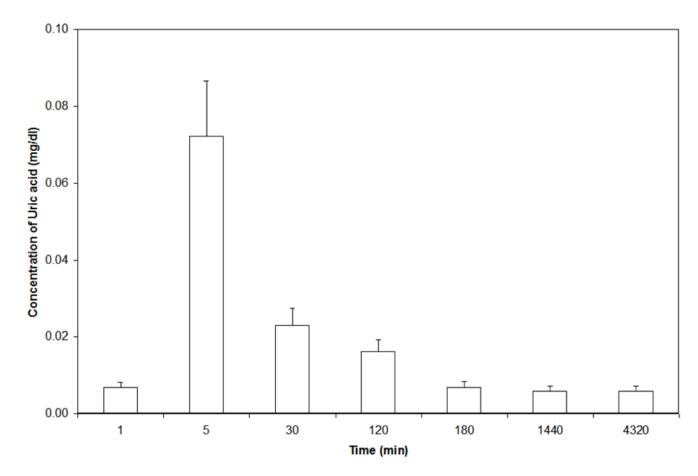


Figure 4.Time-dependent production of UA in kidney tubular cells after treatment with 5 mM fructose.

Table 1 Linear regression lines, correlation coefficients (r), and recovery of UA in urine, plasma, and cell lysates.

Urine 0.0–16.0 y = 3.983x + 0.0602 0.9999 92.81 – 99.62 Plasma 0.0–16.0 y = 3.9911x + 0.0899 90.10 – 103.59 Cell lysate 0.008–4.000 y = 0.6026x – 0.2079 1.0000 98.74 – 106.12	Matrixes	Range of Conc. (mg/dl)	$\mathrm{Equation}^a$	Correlation coefficients (r)	Recovery (%)
$0.0-16.0 \qquad y = 3.983x + 0.0602 \qquad 0.9999 \qquad 9 = 3.981x + 0.0899 \qquad 0.9998 \qquad 9 = 3.9911x + 0.0899 \qquad 0.9998 \qquad 9 = 0.008-4.000 \qquad y = 0.6026x - 0.2079 \qquad 1.0000 \qquad 9 = 0.008-4.000 \qquad 0.008-4.0$					
0.0-16.0 $y = 3.9911x + 0.0899$ $0.008-4.000$ $y = 0.6026x - 0.2079$ 1.0000	Urine	0.0-16.0	y = 3.983x + 0.0602	66660	92.81 – 99.62
0.008-4.000 $y = 0.6026x - 0.2079$ 1.0000	Plasma	0.0–16.0	y = 3.9911x + 0.0899	0.9998	90.10 - 103.59
	Cell lysate	0.008-4.000	y = 0.6026x - 0.2079	1.0000	98.74 - 106.12

 $[\]frac{a}{x}$ and y are the peak area ratios and analytes concentration in a sample, respectively.

HIN YA-HIN Table 2

The intraday and interday assays for UA in urine, plasma, and cell lysates.

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			Intraday (n=5)			Interday (n=5)	
Matrixes	Spiked UA ^a	Mean ± SD	C.V. (%)	RE (%)	Mean ± SD	C.V. (%)	RE (%)
Urine	1.00	1.00 ± 0.05	3.07	0.38	0.97 ± 0.02	1.49	3.40
	4.00	3.82 ± 0.11	2.43	4.46	3.72 ± 0.13	3.03	7.03
	16.00	14.85 ± 0.29	1.87	7.19	14.38 ± 0.55	3.66	10.11
Plasma	1.00	1.04 ± 0.17	2.94	3.59	0.97 ± 0.07	1.27	3.36
	4.00	3.68 ± 0.14	1.69	7.93	3.60 ± 0.30	3.68	9.90
	16.00	15.04 ± 0.35	1.80	5.98	14.48 ± 0.55	2.91	9.52
Cell	8.40^{b}	8.91 ± 0.08	0.16	6.21	8.91 ± 0.06	0.10	5.01
	16.81^{b}	16.47 ± 0.12	0.10	2.12	16.64 ± 0.09	0.07	1.45
	84.06^{b}	84.06 ± 0.01	0.01	0.03	84.06 ± 0.01	0.01	0.02
	1.00^{c}	1.05 ± 0.02	1.30	4.63	1.06 ± 0.02	1.84	6.12
	4.00^{c}	4.05 ± 0.10	2.47	1.27	4.05 ± 0.08	1.19	1.32

 $^{^{}a}$ Unit is mg/dl in the cases of urine and plasma.

 $[^]b$ Unit is $\mu \mathrm{g/dl}.$

 $[^]c$ Unit is mg/dl.