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Simultaneous determination of uric acid metabolites allantoin, 6-aminouracil, and triuret in human urine using liquid chromatography–mass spectrometry

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Abstract

Uric acid (UA) can be directly converted to allantoin enzymatically by uricase in most mammals except humans or by reaction with superoxide. UA can react directly with nitric oxide to generate 6-aminouracil and with peroxynitrite to yield triuret; both of these metabolites have been identified in biological samples. We now report a validated high-performance liquid chromatography and tandem mass spectrometry method for the determination of these urinary UA metabolites. Urine samples were diluted 10-fold, filtered and directly injected onto HPLC for LC–MS/MS analysis. The urinary metabolites of UA were separated using gradient HPLC. Identification and quantification of UA urinary metabolites was performed with electrospray in positive ion mode by selected-reaction monitoring (SRM). Correlation coefficients were 0.991–0.999 from the calibration curve. The intra- and inter-day precision (R.S.D., %) of the metabolites ranged from 0.5% to 13.4% and 2.5–12.2%, respectively. In normal individuals ($n = 21$), urinary allantoin, 6-aminouracil and triuret, were 15.30 (± 8.96), 0.22 (± 0.12), and 0.12 (± 0.10) $\mu\text{g}/\text{mg}$ of urinary creatinine (mean ($\pm$ S.D.)), respectively. The new method was used to show that smoking, which can induce oxidative stress, is associated with elevated triuret levels in urine. Thus, the method may be helpful in identifying pathways of oxidative stress in biological samples.

Keywords

Metabolites; Allantoin; 6-Aminouracil; Triuret; LC; MS/MS; Uric acid

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1. Introduction

Uric acid (UA) is an enzymatic end product of endogenous and dietary purine nucleotide metabolism [1] and a potent antioxidant and scavenger of singlet oxygen and radicals in humans [2]. Markedly increased levels of UA are known to cause gout and nephrolithiasis, but more importantly have been associated with increased risk for the development of cardiovascular disease, particularly hypertension, obesity/metabolic syndrome, and kidney disease [3–7]. UA is converted to allantoin by the enzyme uricase in most mammals, but humans lack this enzyme and therefore have higher plasma levels.

Many authorities consider UA a metabolic end product of purine metabolism that is excreted unchanged in the urine. However, UA can also react with a variety of substances that can lead to its stepwise degradation. Thus, UA is an antioxidant that can react with O_2^- , H_2O_2 , PN ($OONO^-$), and nitric oxide (NO) [8–10], and this may be particularly relevant in the water-soluble fraction within the cytoplasm.

Unlike many antioxidants, the reaction of UA with an oxidant results in its stepwise degradation into a number of end products, and UA cannot be renewed once degraded. The best-known end product is allantoin, which has been shown to be increased in the plasma of subjects with exercise induced oxidative stress [11] or in subjects with renal failure or diabetes [12,13]. Serum allantoin and/or the allantoin/uric acid ratio is also elevated in various diseases such as Down's syndrome [14], chronic venous ulcers [15], and chronic lung disease [16] and has been suggested to be a biomarker for superoxide anion-associated oxidative stress. [17,18].

Recently other uric acid reaction products have also been identified (Fig. 1). For example, Robinson et al. identified triuret as a product of peroxynitrite-mediated oxidation of urate in vitro [19]. We have also identified 6-aminouracil as a product that results from a direct reaction of uric acid with nitric oxide [20]. Until now there are no published methods for the analyses of 6-aminouracil and triuret in biological samples. This could be important if uric acid reactions with NO or peroxynitrite may have a role in cardiovascular disease. We therefore report a validated, sensitive and specific method for their measurements in urine.

2. Experimental

2.1. Chemicals

Standard chemicals were allantoin, 6-aminouracil (6AU), triuret, and creatinine, which were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A). $^{13}C_9$, ^{15}N -Tyrosine (>98% atom ^{13}C , >98% atom ^{15}N) and D_3 -creatinine (98% atom D) were used as internal standards (ISTD) and were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A). Formic acid for buffer solution was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A). Trichloroacetic acid (TCA) was purchased from LabChem Inc (Pittsburgh, PA, U.S.A). All solvents used were HPLC grade of a high purity. Deionized water was distilled before passing through Millipore water purification system. The sample was filtered through a 0.2 μm Nylon micro-centrifuge filter (COSTAR Corning Inc, NY, U.S.A).

2.2. Preparation of standard solutions

Each stock solution of allantoin, 6AU, and creatinine was prepared at concentrations of 10 mmol/L water. The working solutions of various concentrations (1.0, 10.0 and 100.0 $\mu mol/L$) were prepared with water. In the case of triuret, concentrations of stock and working solutions were the same as that used for allantoin and were prepared with 0.5 M NH_4OH . All working solutions were kept below 4 $^{\circ}C$ and all stock solutions were aliquoted and frozen until use. The ISTD was prepared by diluting a $^{13}C_9$, ^{15}N -tyrosine stock solution at 1000.0 $\mu mol/L$ with

methanol/water (1:1, v/v) containing 2.5% (w/v) TCA and a 5.2 mmol/L solution of D₃-creatinine in methanol/water (1:1, v/v) containing 1.0% (v/v) acetic acid.

2.3. Sample preparation for the analysis of UA metabolites

Urine samples were stored at −80 °C before use. For the analyses of metabolites of UA, urine samples were diluted 10 times its volume in distilled water. 200 µL of diluted urine was added to 10 µmol/L of ¹³C₉, ¹⁵N-tyrosine (ISTD) and transferred into an Eppendorf tube. This solution was centrifuged at 14,000 rpm (RCF = 18,078 g) for 10 min (2 times). The supernatant was transferred into micro-centrifuge filter and centrifuged at 14,000 rpm (RCF = 18,078 g) for 5 min, and the filtrate was injected into LC–MS.

2.4. Instrumentation

For the analysis of UA metabolites, a ThermoFinnigan Surveyor liquid chromatography system (ThermoFinnigan, San Jose, CA, USA) and a TSQ Quantum Discovery mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI interface operated in the positive-ion detection mode was used. Nitrogen was used as the sheath and auxiliary gas and set to 40 and 15 (arbitrary units), respectively. The heated capillary temperature was maintained at 300 °C. The collision pressure was 1.5×10^{-3} Torr. The operation of the LC–MS and data analyses was performed using the ThermoFinnigan Xcalibur 1.4 software.

2.5. HPLC–MS/MS conditions

Liquid chromatography analyses were performed in a gradient elution mode using a Phenomenex (Torrance, CA, USA) Luna 5 µ C18(2) 100 Å column, 150 mm × 4.6 mm, coupled with a Phenomenex Luna C18 (2), 5 µm particle size guard column and aqueous and organic mobile phases. The mobile phases used included 0.1% formic acid (A) and methanol (B), in the gradient mode. The nominal column flow rate was 0.6 mL min^{−1} and the flow was split (1:3) prior to the MS. The injection volume was 20 µL. The gradient began at 95% A. Composition was linearly ramped to 25% B over the next 4.5 min, remained constant for 1.5 min, then reversed to the original composition of 95% A over 0.5 min, after which it was kept constant for 0.5 min to re-equilibrate the column. Total run time was 7 min. Allantoin, 6AU, and triuret were analyzed in the positive ESI mode with collision energy (CE) of 25 V. The monitored ions for SRM were as follows: (1) the parent ion of allantoin was *m/z* 158.9 and monitored SRM ions were *m/z* 99.1, 73.1 (quantification ion), and 61.2, (2) the parent ion of 6AU was *m/z* 127.9 and the monitored SRM ions were *m/z* 67.8 (quantification ion) and 84.8, (3) the parent ion of triuret was *m/z* 146.9 and the monitored SRM ions were *m/z* 103.9, 86.9 and 70.0 (quantification ion). In case of ¹³C₉, ¹⁵N-tyrosine as the ISTD for metabolites, the parent ion was *m/z* 191.9 and monitored SRM ions were *m/z* 145.1, 127.0 (quantification ion), and 98.1. Creatinine and D₃-creatinine were detected positive in ESI mode and monitored ions for SRM were as follows; (1) the parent ion of creatinine was *m/z* 113.9 and monitored SRM ions were *m/z* 86.0 (quantification ion) and 72.1 at CE 25V, (2) the parent ion of D₃-creatinine as the ISTD was *m/z* 116.9 and monitored SRM ions were *m/z* 89.0 (quantification ion) and 75.1 at CE 25V.

2.6. Calibration

The quantification of UA metabolites in urine was determined against a calibration curve prepared in water. The calibration curves consisted of the following: (1) standards such as allantoin and triuret concentration levels of 6.0, 12.0, 25.0, 50.0, 80.0, 140.0, and 400.0 µmol/L and (2) 6AU standard concentration levels of 0.2, 0.5, 2.0, 5.0, 25.0, 50.0, and 100.0 µmol/L. In addition, the same calibration standards were prepared in normal urine and these values were calculated from blank normal urine. 20 µmol/L of ¹³C₉, ¹⁵N-tyrosine was added to each sample as the ISTD. In case of creatinine, standard concentrations were 0.5, 1.0, 2.0, 2.5, and

5.0 mmol/L and D₃-creatinine as the ISTD added 0.5 mmol/L. The calibration curves were plotted as concentration vs. peak area ratio and compared to between IS and the analytes.

2.7. Validation of the analytical method

The precision of the method was determined by calculating the intra- and inter-day relative standard deviations (R.S.D., %) for UA metabolites. Intra- and inter-day precisions were measured using the same urine and water stock solutions prepared for calibration curves. To measure the intra-day variance, five sets of prepared samples at each concentration level plus fixed internal standards were generated by using the urine and water stock solutions. The intra-day variance was calculated based on the four or five trial measurements accomplished in the first day, and the inter-day variance was calculated based on the results of five trial analyses carried out on consecutive days. Accuracy of the method was determined by comparing measured concentrations with those of added concentrations, and was expressed as coefficient of variation (CV, %) (percentage difference between the measured and added concentrations).

In case of the metabolites validation, we subjected standard solutions containing allantoin and triuret at 6.0–400.0 µmol/L and 6AU at 0.2–100.0 µmol/L concentrations to determine the detection limits. Calibration samples were prepared as described above and a fixed amount (20 µmol/L of ¹³C₉, ¹⁵N-tyrosine) of ISTD in the same manner in triplicate. Creatinine validation was performed within the range of 0.5–5.0 mmol/L and included ISTD at 0.5 mmol/L. To assess the precision of the method, we prepared the samples for intra- and inter-day R.S.D. %. The accuracy of the method was determined by comparing measured concentrations with those added concentrations (CV, %) and recovery was calculated individually in all calibrators. The recovery test was conducted using corresponding concentration of metabolites spiked in water and compared with those in urine samples. The precision and accuracy estimates had to be within 15% for the assay to be considered acceptable.

2.8. Application to clinical studies

Subjects who provided samples included 21 healthy subjects from Vanderbilt University (Dr. Nancy J. Brown) and 21 healthy non-smokers and 6 smokers from the University of Florida. The study was approved by the local Institutional Review Boards of Vanderbilt University and the University of Florida.

3. Results

3.1. Chromatography and Mass spectra

UA metabolites were readily detected and quantitated under the experimental conditions developed for this study and the total ion chromatograms (TIC) are shown in Fig. 2. Allantoin, 6AU and triuret were measured in the positive ESI mode. All of the metabolites were well separated from each other with this chromatography method.

Positive ion mode was used for the analysis UA metabolites in the full scan, MS/MS and SRM methods. The [M–H]⁺ ion of ¹³C₉, ¹⁵N-tyrosine, the IS, was *m/z* 191.9 and it fragmented to *m/z* 145.1 ([M–¹³COOH]⁺), 127.0 ([M–¹³COOH¹⁵NH₂]⁺), and 98.1 ([¹³C₇H₈]⁺). Although the major product ion for allantoin was *m/z* 99.1 [M–NH₂CONH₂]⁺ the product ion [CONHCO]⁺ at *m/z* 73.1 was used for quantitation because it was completely separated from urine background and yielded a better signal-to-noise ratio. 6AU (*m/z* 127.9 [M–H]⁺) produced fragments at *m/z* 84.8 [M–NHCO]⁺ and at *m/z* 67.8 [M–NHCO–NH₃]⁺, the latter of which was used for quantitation. 6AU is present in lower concentrations than allantoin in normal urine and the limit of quantitation (LOQ) was 0.2 µmol/L. Triuret (*m/z* 146.9 [M+H]⁺) produced ions of at *m/z* 129.8 [M–NH₃]⁺, and at *m/z* 103.9 [M–CONH]⁺, *m/z* 86.9 [M–CONH–NH₃]⁺, and *m/z* 70.0. Although the *m/z* 70.0 ion is less abundant than the other SRM ions, it is very

well separated from background peaks in urine. The LOQ of triuret was 6.0 $\mu\text{mol/L}$. Due to changes in ambient HPLC conditions, slight changes in the retention times of $^{13}\text{C}_9$, ^{15}N -tyrosine (from 5.11 to 5.29 min) and triuret (from 5.30 to 5.37 min). This did not affect the identification or quantitation which is based on LC–MS/MS. In this study, all of metabolites of uric acid could be detected under the experimental conditions.

3.2. Precision, accuracy and recovery

Linear calibration curves were obtained for each of the metabolites; the correlation coefficients and equations for the standard curves are shown in Table 1. The intra- and inter-day precision (R.S.D., %) for all metabolites was between 0.7% and 13.4%. There was a linear response for the UA metabolites in the range of 0.2–400.0 $\mu\text{mol/L}$ with correlation coefficients ranging from 0.991 to 0.999 in urine. The recovery and intra- and inter-assay relative standard deviations are shown in Table 2. The accuracy (CV, %) ranged from 0.2% to 13.8%. Recovery ranged between 87.5% and 125.1%. The precision (R.S.D. %) was 0.5–13.4% for three different runs. The accuracy and precision for creatinine were 0.1–3.5% and 0.3–5.0% intra-assay and 0.3–1.0% and 0.6–7.3% inter-assay, respectively. Recovery of creatinine was between 92.0% and 103.8%.

3.3. Application in normal urine samples

The method developed was successfully used to measure UA metabolites in urine. Allantoin excretion was higher compared with other metabolites such as 6AU and triuret in human normal urine (Fig. 3). Urinary levels of 6AU and triuret were detected at low levels in normal human urine samples.

3.4. Application to pathological conditions: effect of smoking

The urinary concentration of triuret was compared in subjects who are non-smokers with those who are heavy smokers. Triuret was detected at higher concentrations in the urine of smokers compared to normal healthy volunteers. Fig. 4 compares a normal male smoker compared with a non-smoking male (Fig. 4) and Fig. 5 shows the mean levels of urinary triuret in smokers versus non-smoking control subjects.

4. Discussion

Uric acid is converted to allantoin by enzymatic and electrochemical oxidation *in vitro* [22] and *in vivo* [23,24]. While assays for allantoin have been reported [23–25], in this study we developed a new method for the analyses and quantification of allantoin along with other UA metabolites in human urine. Allantoin is stable and not affected by pH of standard solutions and urine samples [26]. Since allantoin is a very polar compound, some groups have used a polymeric amino column to retain allantoin and thereby facilitate its isolation [27]. For pre-column derivatization, reagents such as 2,4-dinitrophenyl hydrazine (DNPH) and 7-fluoro-4-nitrobenzoxadiazole (NBD-F) were used as biogenic amine compounds [25,26]. These derivatizations can be tedious and time-consuming. In this study, a sensitive and selective analytical method using LC–MS/MS was developed. Allantoin has the fragmentation at m/z 99.1 as a major ion and at m/z 73.1 as a minor ion under the collision energy of 25V condition in ESI positive mode. For the quantification from urine sample, we compared m/z 99.1 and m/z 73.1. The m/z 99.1 was a nice peak shape in standard solution whereas m/z 99.1 peak had a poor shape and poor signal-to-noise (S/N) in urine samples. The ion m/z 73.1 was also separated from nearby background peaks and had a good value of 292 as the S/N (data not shown). As shown in Fig. 3, allantoin was detected easily and urine matrix backgrounds did not interfere in this method.

Recently two other potentially significant biological metabolites of uric acid have been identified as 6AU and triuret [20,21]. 6AU is produced from uric acid and nitric oxide whereas triuret is derived from the chemical reaction of uric acid with peroxynitrite [19]. To develop a simultaneous analytical method for 6-aminouracil and triuret, we performed a simple preparation step of urine samples and developed a rapid and sensitive analysis method using LC–MS/MS. As shown, 6-aminouracil and triuret had a good limit of detection of approximately 0.2 $\mu\text{mol/L}$ at a (S/N) = 5 and a level of 0.2 $\mu\text{mol/L}$ at a (S/N) = 10, individually. The accuracy and precision were acceptable at 1.0–13.8% and 0.7–4.8% for 6-aminouracil and 0.2–8.0% and 2.7–9.9% for triuret, respectively.

The concentration of UA in urine is about 1.49–4.46 mmol/L in healthy people [28]. However, the normal concentration values of UA metabolites in healthy smoking and non-smoking humans are not known. We were able to detect allantoin, 6AU, and triuret in human urine with the method developed. Allantoin concentration was 15.30 (± 8.96) (varied from 3.50 to 35.86) $\mu\text{g/mg}$ creatinine in human urine and 6AU was present in a concentration of 0.22 (± 0.12) ($\mu\text{g/mg}$ creatinine. The absolute concentrations that were measured for allantoin and 6AU were 8.05 (± 2.27) and 0.22 (± 0.13) $\mu\text{mol/L}$, respectively. Interestingly, triuret was minimally present (Fig. 3 and 0.078 $\mu\text{mol/L}$ in Fig. 4C) and was below the limit of detection (0.2 $\mu\text{mol/L}$) in many cases of urine from healthy, non-smokers. In contrast, triuret was detected in the urine of 6 heavy smokers (up to 0.580 $\mu\text{mol/L}$, mean 0.380 $\mu\text{mol/L}$ in 6 smoker samples) as shown in Figs. 4A and 5. This suggests that the oxidative stress associated with smoking may have generated peroxynitrite that could react with uric acid to form triuret. Indeed, smoking can result in peroxynitrite generation [29,30] and can also acutely reduce serum uric acid levels [31].

5. Conclusion

We have successfully developed a method for analyzing UA metabolites using LC–MS/MS in human urine. Each of these metabolites is a signature end product reflecting distinct pathways for UA degradation in the presence of different oxidants. Measurements of these metabolites in biological samples may pave the way for determining the significance of each of the pathways in human pathology.

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Fig. 1.
The pathway and structures of UA and its metabolites.

Fig. 2. Extracted SRM chromatograms of UA metabolites standards spiked in water. All of them were detected in ESI positive mode. (A) 6AU (6-aminouracil, 0.2 $\mu\text{mol/L}$, 4.13 min), (B) triuret (6.0 $\mu\text{mol/L}$, 5.37 min), (C) allantoin (6.0 $\mu\text{mol/L}$, 2.83 min), and (D) $^{13}\text{C}_9$, ^{15}N -tyrosine (20.0 $\mu\text{mol/L}$, 5.29 min, ISTD).

Fig. 3.

Chromatograms of the detected UA metabolites in urine sample from healthy volunteer. Urine samples were diluted 10-fold and filtered as described. (A) 6AU (RT: 4.14 min, S/N 83, 2.49 $\mu\text{mol/L}$), (B) triuret (not detected in urine from healthy, non-smokers), (C) allantoin (RT: 2.85 min, S/N 203, 3.10 $\mu\text{mol/L}$), and (D) $^{13}\text{C}_9, ^{15}\text{N}$ -tyrosine (RT: 5.29 min, S/N 3659, IS). The value is absolute value.

Fig. 4. Chromatograms of triuret detected in urine from a heavy smoker and non-smoker. All urine was diluted 10-fold. (A) Triuret is present in the urine of a smoker, (B) ISTD in the urine of a smoker, (C) triuret in the urine of a non-smoker, (D) ISTD in urine from non-smoker. ISTD is $^{13}\text{C}_9,^{15}\text{N}$ -tyrosine.

Fig. 5. Triuret concentrations in the urine samples from smoker ($N = 6$, 0.34 ± 0.04 µg/mg creatinine) and non-smoker healthy volunteers ($N = 21$, 0.12 ± 0.10 µg/mg creatinine).

Table 1Linear regression lines and coefficients correlation (*r*) of UA metabolites in urine.

Analytes	Range of conc. (μmol/L)	Equation ^a	Correlation coefficient (<i>r</i>)
Allantoin	6.0–400.0	$y = 0.0061x - 0.0003$	0.9999
6-Aminouracil	0.2–100.0	$y = 0.1515x - 0.0372$	0.9991
Triuret	6.0–400.0	$y = 0.0003x - 0.0004$	0.9911

^a *x* and *y* are the peak area ratios and analytes concentration (μmol/L) in a sample, respectively.

Table 2
Precision and accuracy of the determination of UA metabolites in human urine.

Analytes (μmol/L)	Intra-day (n = 5)		Inter-day (n = 5)			
	Mean ± S.D.	R.S.D. (%)	CV (%)	Mean ± S.D.	R.S.D. (%)	CV (%)
Allantoin						
6.0	5.8 ± 0.4	6.4	4.0	5.9 ± 0.3	5.4	1.8
12.0	12.8 ± 1.7	13.4	7.1	12.4 ± 1.3	10.3	3.1
25.0	24.1 ± 1.9	7.8	3.7	26.0 ± 3.2	12.2	4.0
50.0	54.9 ± 1.8	3.3	9.7	49.0 ± 4.6	9.5	2.0
80.0	85.1 ± 3.5	4.2	6.4	80.7 ± 4.3	5.3	0.8
140.0	137.4 ± 1.3	1.0	1.8	410.8 ± 9.7	6.9	0.6
400.0	373.1 ± 8.6	2.3	6.7	392.6 ± 12.9	3.3	1.9
6AU						
0.2	0.2 ± 0.01	2.7	7.3	0.2 ± 0.01	2.5	3.5
0.5	0.4 ± 0.01	2.8	13.8	0.5 ± 0.03	5.7	8.2
2.0	1.2 ± 0.04	1.9	2.4	2.0 ± 0.07	3.7	0.3
5.0	5.1 ± 0.04	0.8	1.0	4.9 ± 0.32	6.5	2.6
25.0	27.4 ± 1.31	4.8	9.6	26.7 ± 1.84	6.9	6.7
50.0	51.7 ± 0.36	0.7	3.4	51.3 ± 2.09	4.1	2.6
100.0	104.3 ± 0.97	0.9	4.3	104.3 ± 3.53	3.4	4.3
Triuret						
6.0	5.8 ± 0.3	5.2	3.0	5.8 ± 0.4	6.1	2.9
12.0	12.1 ± 1.2	9.9	0.8	11.6 ± 0.8	6.7	3.3
25.0	26.2 ± 1.8	7.0	4.9	26.0 ± 0.8	3.3	4.1
50.0	54.0 ± 3.2	6.0	8.0	55.5 ± 4.7	8.5	11.0
80.0	80.1 ± 2.1	2.7	0.2	81.7 ± 3.6	4.4	2.2
140.0	133.7 ± 7.1	5.3	4.5	138.4 ± 4.2	3.0	1.2
400.0	391.5 ± 11.4	2.9	2.1	377.5 ± 24.8	6.6	5.6