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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) μg/mg of urinary creatinine (mean (±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.

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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

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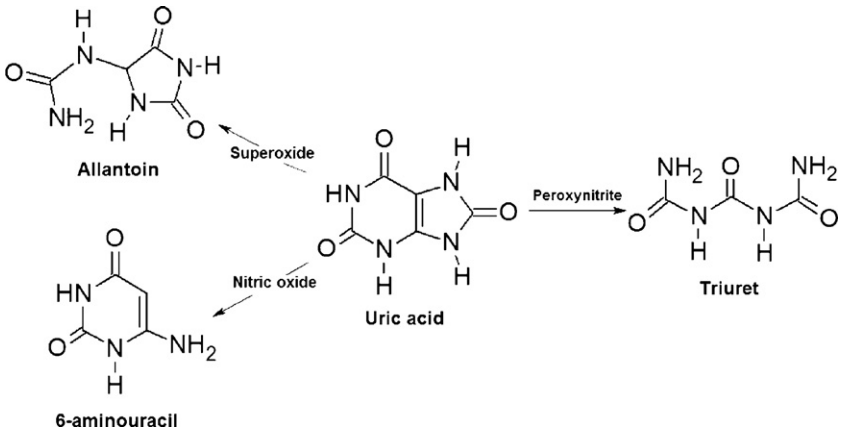

Fig. 1. The pathway and structures of UA and its metabolites.

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 μmol/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 °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 μmol/L with methanol/water (1:1, v/v) containing 2.5% (w/v) TCA and a 5.2 mmol/L solution of D_3-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 $^{13}C_9$,^{15}N-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 $^{13}C_9$,^{15}N-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 D_3-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 D_3-creatinine as the ISTD was *m*/*z* 116.9 and moni-