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Allantoin in Human Urine Quantified by UPLC-MS/MS

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Abstract

Uric acid is a potent antioxidant and scavenger of singlet oxygen and other radicals in humans. Allantoin, the predominant product of free radical-induced oxidation of uric acid is efficiently excreted in the urine and has potential as a biomarker of oxidative stress. We developed a rapid and specific assay for urinary allantoin using ultra performance liquid chromatography-tandem mass spectrometry suitable for high-throughput clinical studies. The method required minimal sample preparation, was accurate (mean error 6%), precise (intra- and inter-day imprecision: <8%) and sensitive (limit of detection: 0.06 pmol). Allantoin levels measured in control samples were comparable to literature values.

Keywords

Oxidative stress; reactive oxygen species; allantoin; UPLC-MS/MS

Oxidative damage occurs when there is an imbalance between the production and elimination of reactive oxygen species (ROS). When antioxidant defense systems are unable to balance the deleterious effects of ROS, indiscriminant damage occurs to biological molecules such as DNA, lipids, and proteins [1]. Measurement of ROS *in vivo* presents a challenge, because these molecules are short lived and cannot be directly detected in humans [2]. An alternative approach is to measure biomarkers that are the products of reactions between biological molecules and ROS. Several molecules have been identified as relevant biomarkers of oxidative status and significant attempts are being made to develop assays to quantify these biomarkers in biological fluids [3]. Uric acid, the terminal product of purine metabolism in humans, is a potent antioxidant and scavenger of reactive oxygen species [4]. Allantoin is the predominant product of these oxidative reactions [2; 5; 6] and was shown to be stable in urine [7]. Importantly, allantoin reflects the systemic level ROS independently of uric acid levels [8; 9; 10]. In the urine of healthy individuals, its levels vary at least two-fold [10; 11; 12], providing an opportunity to rank individuals with regard to their presumed oxidative status by urinary allantoin levels. Thus allantoin presents a promising biomarker for monitoring oxidative status in humans. Cross-sectional studies have demonstrated increased levels of allantoin in disorders associated with oxidative stress, including diabetes

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[9; 11; 13], inflammatory and autoimmune conditions [5; 14; 15], cardiovascular [8], renal [9], pulmonary [16; 17], and Wilson's disease [18; 19]. Moreover, urinary, plasma, and muscle allantoin were shown to correlate with other markers of oxidative stress associated with exercise [20]. Historically, allantoin has been measured by the colorimetric assay based on the Rimin-Schryver reaction [21] with improvements using HPLC [5]. GS/MS methods have been developed to quantify urinary allantoin [22]. However, these methods are both time-consuming and expensive due to the required derivatization, or they pose a challenge with regards to reproducibility. Several LC-MS/MS techniques also have been developed in recent years [7; 11; 22; 23; 24]. In the most recent study, Kim *et al.* [24] used HPLC and reverse phase as the separation method. The objective of our study was to develop a simple, rapid and more sensitive method with minimal sample preparation using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The advantages of UPLC compared with HPLC are improved resolution and higher sensitivity, which reduce the amount of specimen required for the assay, and significantly reduced analysis time.

Analyses were performed on an Acquity UPLC system and TQD triple quadrupole mass spectrometer equipped with an ESI source (Waters Corporation, Milford, MA). Allantoin standard was purchased from Sigma-Aldrich Corp. (St. Louis, MO) and DL-allantoin-5-¹³C; 1-¹⁵N internal standard (IS) from C/D/N Isotopes (Quebec, Canada). Allantoin calibrants, 1 to 500 $\mu\text{mol/L}$, were prepared using synthetic urine as the matrix. The synthetic urine was prepared according to published method [25], with modifications (see supplemental material). Low and high QCs were derived from pooled urine control samples with appropriate levels of endogenous allantoin. Control ranges were determined using urine specimens collected from a cohort of 45 control subjects who were consented for the study following DUMC IRB procedures. Urine samples were vortex-mixed and centrifuged at 15,000 rpm for 10 minutes to pellet solid matter prior to sample preparation. 25 μL calibrant, QC or urine was added to 25 μL DL-allantoin-5-¹³C;1-¹⁵N IS solution (100 μM) prepared in DI-H₂O and 450 μL matrix (0.5% formic acid in acetonitrile: DI-H₂O (95:5, v/v)). The samples were vortex-mixed, centrifuged at 15,000 rpm for 10 minutes and 5 μL of sample was injected onto an Acquity UPLC™ BEH HILIC, 1.7 μm , 2.1 $\times$ 100 mm column (Waters Corp., Milford, MA) heated to 40°C. Chromatographic separation was achieved by isocratic elution using 0.5% formic acid in acetonitrile: DI-H₂O (95:5, v/v) as the mobile phase, with a flow rate of 200 $\mu\text{L/min}$. Allantoin and the IS were detected in positive ion mode, using multiple reaction monitoring (MRM). MRM transitions for the protonated molecular ions, $[\text{M}+\text{H}]^+$, of allantoin were 159>116 (primary) and 159>61 (secondary). The corresponding transitions monitored for DL-allantoin-5-¹³C;1-¹⁵N IS were 161>118 and 161>61. The primary transitions were used to quantify the allantoin and secondary transitions were used as qualifier ions. The following mass spectrometer parameters were used: capillary voltage 3.5 kV, cone voltage 19 V, collision energy 8 eV, desolvation temperature 350°C, and argon collision gas flow 0.13 mL/min. The analysis time was 4 minutes. Figure 1 shows a representation of the allantoin pseudomolecular ion $[\text{M}+\text{H}]^+$ in positive ion mode (m/z 159) and its primary fragmentation product. Figure 2 shows MRM chromatograms of allantoin and DL-allantoin-5-¹³C;1-¹⁵N IS in a standard and a control urine sample. The chromatographic conditions enabled the resolution of allantoin from an interfering peak in urine that eluted immediately prior to DL-allantoin-5-¹³C;1-¹⁵N IS. The retention time for allantoin and for DL-allantoin-5-¹³C;1-¹⁵N IS in urine was 1.9 minutes. The peak area ratio of allantoin to the IS was used to determine absolute concentrations of allantoin, and allantoin was normalized to the creatinine concentration in urine.

The calibration curve was derived from the peak area ratio of the primary MRM transition for allantoin to that of DL-allantoin-5-¹³C;1-¹⁵N IS using TargetLynx® software (Waters Corp., Milford, MA). The curve was linear over the concentration range 1 – 500 $\mu\text{mol/L}$ ($r^2>0.99$) and was stable over 15 weeks (mean $\pm$ SD slope: 0.0172 ± 0.0001 , $n = 7$). The

mean observed error over the calibration range of back-calculated concentrations was between 3% and 18%. Intra-day imprecision, determined by the replicate analysis of control urines with low (mean $\pm$ SD: $86 \pm 1.2 \mu\text{mol/L}$, $n = 11$) and moderate ($158 \pm 1.1 \mu\text{mol/L}$, $n = 9$) endogenous allantoin concentrations was 2% or less. Inter-day imprecision, determined by replicate analyses over an 8 week period was 4% for a low concentration ($89 \pm 3.4 \mu\text{mol/L}$, $n = 9$) and 8% for a high concentration urine sample ($310 \pm 23.5 \mu\text{mol/L}$, $n = 7$). LOQ was defined as the lower limit of calibration ($1 \mu\text{mol/L}$) where accuracy and precision were within acceptable limits ($<20\%$). The limit of detection, defined by a signal to noise ratio of 3 to 1, was 0.06 pmol . The effect of storage temperature and freeze-thaw on the stability of allantoin was investigated. Allantoin stability was assessed using pooled urine samples with low and high concentrations. Allantoin was stable for up to 6 days at RT, up to 12 days at 4°C , at least 15 weeks at -70°C , and after 5 cycles of freeze thaw.

Urinary allantoin levels were determined in control subjects (19 males, 26 females, median age: 35, range: 4-75). The mean concentration was $9.9 \pm 5.3 \text{ mmol/mol CR}$ (range: 3.4 to 26.1 mmol/mol CR) and was comparable to literature value [24]. In the adult population ($>18 \text{ yrs}$) no significant correlation was observed between allantoin levels and age. Mean allantoin levels were 8.0 ± 3.7 and $10.2 \pm 5.4 \text{ mmol/mol CR}$ in adult ($>18 \text{ yrs}$) males ($n = 17$) and females ($n = 22$), respectively, but no significant difference was observed.

In conclusion, we have developed and validated a UPLC-MS/MS method for the determination of allantoin in urine. We used normal phase chromatography which improves sensitivity of the electrospray process by virtue of the high organic solvent content in the mobile phase. Thus this method is very sensitive, requiring a minimal sample volume and has a simple sample preparation procedure and rapid analysis, making it suitable for studies involving large cohorts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CR	Creatinine
DI	De-ionized
ESI	Electrospray ionization
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
QC	Quality control
ROS	Reactive oxygen species
RSD	Relative standard deviation also referred to as CV%
UPLC	Ultra performance liquid chromatography

Figure 1. Representative pseudomolecular ion $[M+H]^+$ structure of allantoin, (m/z 159). Formation of the major fragmentation product (m/z 116) is indicated by the dotted line.

Figure 2.
MRM chromatograms of the primary transitions for allantoin and DL-allantoin -5-¹³C;1-¹⁵N IS in a standard solution (A), and in a control urine (B).