Short communication

Detection of allantoin in clinical samples using hydrophilic liquid chromatography with stable isotope dilution negative ion tandem mass spectrometry

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ABSTRACT

Allantoin is the major oxidation product of urate in humans and is a potential biomarker of oxidative stress. Several methods are used to measure allantoin in biological samples but they have inherent issues that can include lack of specificity and sensitivity, difficulty in sample preparation, or artefactual generation of allantoin. We have developed a method for measuring allantoin using hydrophilic liquid chromatography with stable isotope dilution tandem mass spectrometry (HILIC–MS/MS). It was validated for measuring allantoin in plasma, synovial fluid and urine from human subjects. The limit of quantification was determined to be 10 fmol and the assay displayed excellent linearity for the wide range of concentrations found in clinical samples. Relative standard deviations were <5% for between-day and <7% for within-day variation. Accuracy was between 100% and 104%. Concentrations of allantoin in plasma of healthy controls (2.0 μM; interquartile range 1.4–3.6 μM, $n = 35$) was significantly lower ($p < 0.001$) than that in plasma from patients with rheumatoid arthritis (3.7 μM; IQR 3.0–5.6 μM, $n = 43$) and in synovial fluid of patients with gout (3.3 μM; IQR 2.8–5.8 μM, $n = 10$). This newer HILIC–MS/MS method is a simple and highly sensitive assay for detection of allantoin. It can be used to assess the level of oxidative stress in human pathologies.

1. Introduction

Urate is considered to be end product of purine metabolism in humans. It is found at significant levels in body fluids and is frequently observed above 400 μM in plasma [1], synovial fluid [2], and epithelial lining fluid in the lungs [3]. Urate is a facile reducing agent and is oxidised by a number of different reactive oxygen species including hydroxyl radical, peroxynitrite, and hypochlorous acid [4]. In addition, we recently showed that urate is a physiological substrate for neutrophil enzyme myeloperoxidase [5]. In all cases, allantoin is the major stable oxidation product of urate. Consequently, allantoin is a potentially useful non-specific biomarker of oxidative stress [6–8]. However, it has been difficult to detect allantoin in plasma and urine because it is present at relatively low concentrations, has no useful chromophore, and its high polarity means it is poorly retained by reversed phase chromatography.

Until recently, allantoin was detected via conversion to glyoxylic acid with subsequent derivatisation with dinitrophenyl-hydrazine and then measured using high-performance liquid chromatography (HPLC) with UV detection [9,10]. Various gas chromatography–mass spectrometry (GC/MS) methods have been developed in recent times to detect allantoin in clinical samples. These methods have given variable results. For example, measurement of allantoin concentration in serum from healthy controls has ranged from 8 to 40 μM [8,11–14]. This variation has potentially arisen from the prolonged exposure of urate to sodium hydroxide, which facilitates its oxidation to allantoin [15,16]. A more recent GC/MS method has been developed that avoids prolonged exposure to sodium hydroxide [17]. Using this method, the allantoin levels detected in plasma were found to be much lower than previously reported with a mean of 2.4 μM with 5–95% percentiles of 0.9–8.0 μM. A significant limitation of this newer GC/MS method is that it involves a time-consuming derivatisation step. Capillary electrophoresis and an enzymatic method gave concentrations of allantoin in healthy controls of 3.5 μM (S.D. 0.96) [18] and 8.2 μM (S.D. 3.1) [19], respectively.

A survey of the literature shows that either normal phase chromatography [19] or hydrophilic interaction liquid chromatography (HILIC) [20,21] are the most suitable methods for retaining

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allantoin so that it is amenable to selective and sensitive analysis. HILIC is a relatively new chromatographic technique that enables the retention of highly polar compounds, such as allantoin, that are not retained by reverse phase C18 columns. HILIC also has the advantage that analytes are run in predominantly organic solvent such as acetonitrile that are more readily ionisable than water based solvents [20]. This means that lower detection limits are often achieved when using a triple quadrupole mass spectrometer. In the current study we have combined the advantages HILIC and stable isotope dilution negative ion tandem mass spectrometry to develop a sensitive and specific method to quantify allantoin in biological samples.

2. Experimental

2.1. Chemicals

Allantoin, ammonium acetate and uric acid were from Sigma (St. Louis, MO, USA). Labelled allantoin (^{15}N) was purchased from Icon Isotopes (Summit, NJ, USA) and acetonitrile was from Malinckrodt Baker (Paris, KY, USA). Water of 18 MΩ quality was prepared from a Millipore Milli-Q system (Bedford, MA, USA).

2.2. Preparation of standards

Allantoin standards were prepared in the starting solvent mixture (90% acetonitrile/10% water). The standards were prepared in a range from 25 nM to 20 μM for plasma and synovial fluid samples with 1.1 μM of internal standard (^{15}N allantoin). The range of allantoin standards for human urine was from 1 μM to 60 μM with internal standard present at a concentration of 11 μM. These concentrations ranges were chosen to match that expected in the clinical samples after they had been diluted 10-fold to precipitate proteins (see below). Calibration curves were plotted with the y-axis representing the ratio of peak areas for ^{14}N allantoin and the isotopically labelled ^{15}N allantoin while the x-axis representing the amount of ^{14}N allantoin. Uric acid (10 mM) was dissolved in sodium hydroxide at pH 11 and then glacial acetic acid was immediately added to drop the pH to 7.4 and minimise artefactual oxidation of urate.

2.3. Chromatographic conditions

Allantoin was separated by liquid chromatography using a TSK Gel-Amide column (Tosoh, Tokyo, Japan; 150 mm × 2.0 mm, 5 μm) held at 30 °C with a Luna silica guard cartridge (Phenomenex, Santa Barbara, USA; 4 mm × 2.00 mm). Eluent A was ammonium acetate (10 mM, pH 6.8) and eluent B was acetonitrile. Initial conditions (10% A) were maintained for 5 min before a linear gradient to 50% A over 5 min was used, maintained at 2 min before returning to starting conditions in 1 min and re-equilibration. The flow rate was 0.2 ml/min and the injection volume was 10 μL. Samples were maintained at 4 °C in the autosampler. The eluent from the column was introduced into the mass spectrometer without splitting and diverted to waste for the first 4 min of the run.

2.4. Mass spectrometry conditions

Mass spectrometry analyses were performed using an Applied Biosystems 4000 QTrap (Concord, ON, Canada). The electrospray needle was held at 400 °C, nitrogen was used for the curtain and collision gases. The ion spray was −4.5 kV and the declustering potential was 78 V. Allantoin was detected and quantified by multiple reaction monitoring (MRM) using negative electrospray ionisation mass spectrometry. For unlabelled ^{12}C allantoin, the largest fragment ion (m/z 114) generated by collision induced

disassociation of the $[M-H]^-$ ion (m/z 157) was used for quantification. A second fragment ion (m/z 97) was used for confirmation (Fig. 1). For ^{15}N labelled allantoin, the transition used for detection and quantification was m/z 159–116. The time allowed for each scan was set to 50 ms. Data acquisition was performed using Analyst 1.4.2.

2.5. Assay validation

Sets of standards and quality controls were prepared and analysed on four different days to assess linearity, accuracy and precision. Recovery was assessed by spiking plasma and urine samples with known concentrations of allantoin. Three plasma samples with relatively low levels of urate (200–225 μM) and known levels of allantoin were spiked with additional urate (400 μM) to check whether allantoin was artefactually produced from urate oxidation during sample preparation.

2.6. Clinical samples

The assay was used to detect allantoin in extracts from plasma, synovial fluid and urine. Samples were obtained from healthy volunteers and patients with either rheumatoid arthritis or gout. Ethical approval for donation of blood, urine and synovial fluid was provided by Upper South Ethics Committee, Christchurch, New Zealand. Protein in samples was precipitated using a nine-fold excess of acetonitrile containing the ^{15}N allantoin internal standard (110 nM for human plasma and synovial fluid and 1.1 μM for human urine; this equated to a final concentration before injection of 100 nM for plasma and 1 μM for urine, respectively). Typically 450 μL of acetonitrile was added to 50 μL of sample. The samples were then centrifuged at 16,000 × g for 5 min and 10 μL of the supernatant was injected onto the LC column.

3. Results and discussion

3.1. Method development

Allantoin was retained on the TSK Gel-Amide HILIC column and had a retention time of approximately 6.3 min (Fig. 2). The peak shape and the sensitivity of the method were optimal when the column was maintained at 30 °C. Addition of allantoin to plasma caused a proportional increase in its signal (Fig. 2A and B). The expected increase in signal also occurred when allantoin was added to urine. Initially positive electrospray ionisation was used for mass spectrometric detection and this was sensitive to 50 nM (not shown). However, there was considerable noise which resulted in a low signal to noise ratio. Switching from positive to negative ion mode improved the sensitivity at least 10-fold. This enabled more accurate detection of allantoin in plasma and synovial fluid.

3.2. Assay validation

The assay was selective for allantoin due to the combination of liquid chromatography separation with an isotopically labelled internal standard and selective monitoring of specific fragment ions of the $[M-H]^-$ ion of allantoin. For a contaminant species to interfere with the analysis of allantoin it would have to elute at an identical retention time, have the same m/z ratio in negative ion mode, and also exhibit the same fragmentation pattern. Linear calibration curves were obtained for allantoin in plasma and urine samples over the concentration ranges tested (Fig. 3). Allantoin was detectable in plasma and urine below 1 μM. Results for accuracy and precision for all QCs are summarised in Table 1. Accuracy was within the range of 100–104% and relative standard deviations were <7% for intra-day precision and for inter-day precision. The
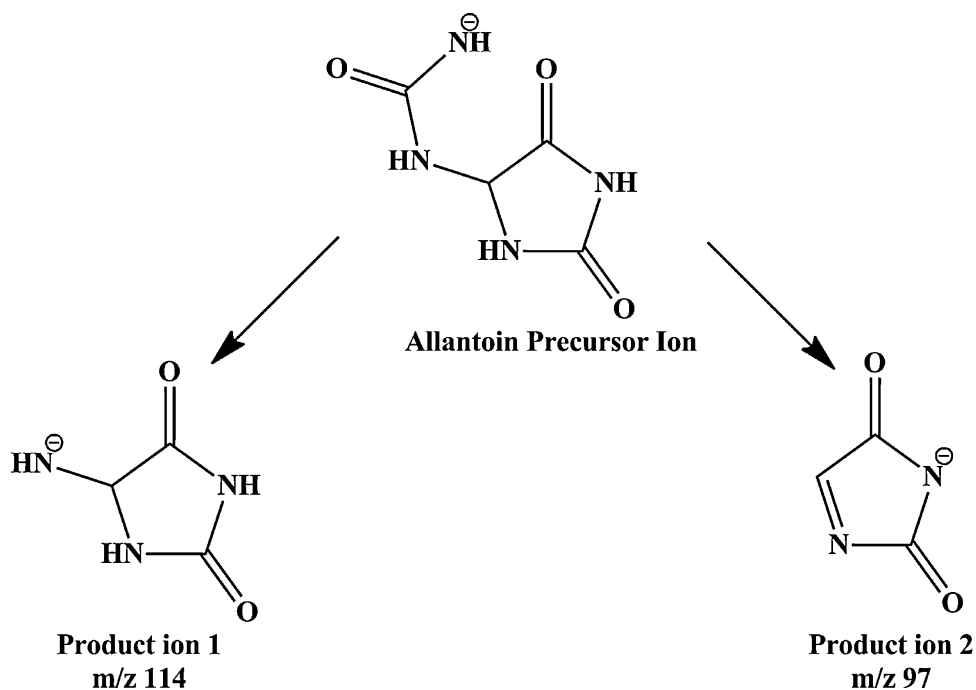
Fig. 1. Structures of the negative precursor ion of allantoin and its fragment ions. The dashed lines on the allantoin indicate the cleavage site when undergoing fragmentation to product ions 1 and 2.
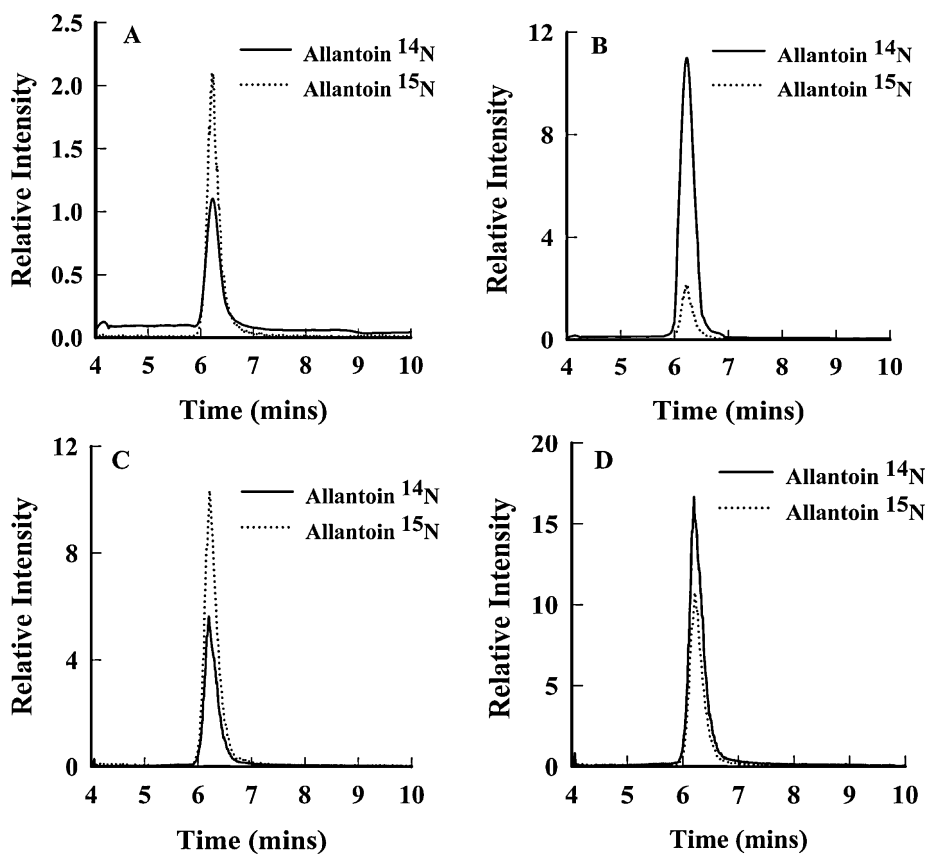

Fig. 2. Multiple reaction monitoring of the ions for allantoin and its internal standard in clinical samples. (A) Human plasma with 1 μM internal standard, (B) human plasma spiked with 5 μM allantoin with 1 μM internal standard, (C) human urine with 5 μM internal standard, and (D) human urine spiked with 5 μM allantoin and 5 μM internal standard.
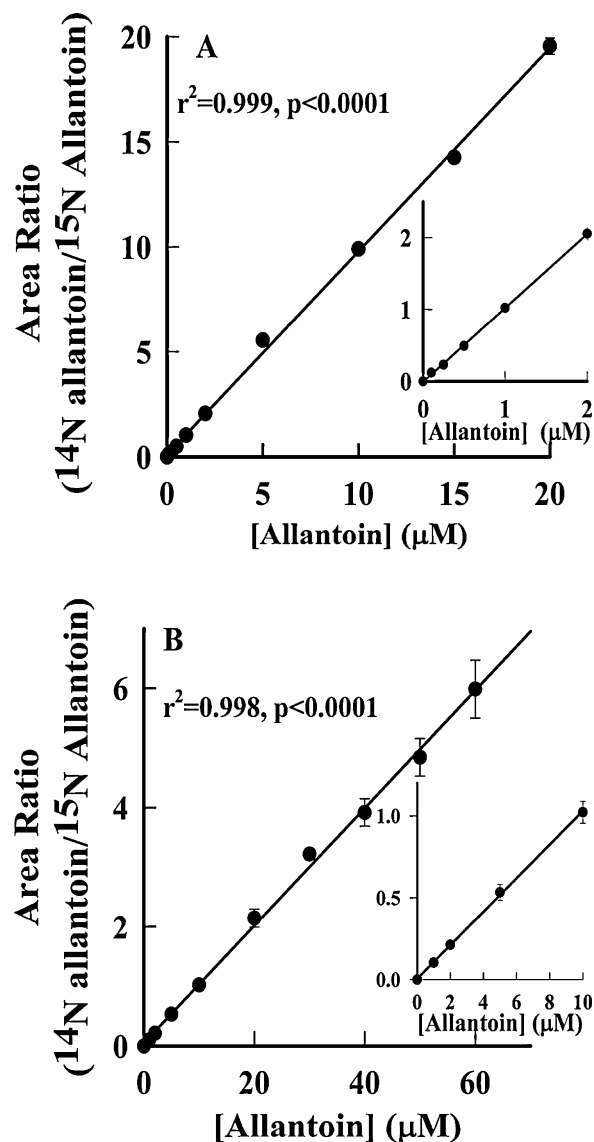
Fig. 3. Calibration curves generated for determination of allantoin in human plasma and synovial fluid (A) and human urine (B). Standards were prepared in 90% acetonitrile and represented the concentration of allantoin in 10-fold diluted plasma, synovial fluid and urine. The isotopically labelled internal standard represented 1 μM in undiluted plasma and synovial fluid and 10 μM in urine, respectively. Each data point represents mean area ratio $\pm$ standard deviation from at least four calibration curves plotted on different days. Where error bars are not visible, they fall within the symbol size.

Table 1
Inter and intra day precision (RSD), accuracy and recovery for allantoin.

	Allantoin		
	Low QC	Medium QC	High QC
Accuracy			
Nominal (μM)	1.00	10.0	50.0
Mean (μM) ($n=5$)	1.02	10.4	50.7
Bias (%)	2.3	3.8	0.8
Precision			
Intra-day RSD (%)	6.3	4.4	2.7
Inter-day RSD (%)	5.5	3.6	4.9

Quality controls were prepared (Low, Med and High QC) covering the expected concentration range in biological samples. To assess accuracy, a nominal amount of allantoin was injected and quantified using the LC–MS/MS method described. Results are calculated from analysing five samples at each nominal concentration. The same quality controls were used to determine precision. RSD; residual standard deviation.

Table 2
Effect of sample matrix on recovery of internal standards and allantoin added to clinical samples.

	Recovery	
	$^{15}N_2$-allantoin	5 μM spiked allantoin
Plasma ($n=6$)	97 $\pm$ 3%	98 $\pm$ 4%
Urine ($n=6$)	101 $\pm$ 4%	96 $\pm$ 2%
Synovial fluid ($n=6$)	98 $\pm$ 2%	Not tested

The average peak area of the isotopically labelled internal standard in clinical samples was compared to that observed in buffer alone.

limit of quantification (S/N > 10) in standard samples was 10 fmol. The limit of detection (LOD; S/N ratio > 3) was not determined but was lower than the LOQ.

Recovery of the internal standard ^{15}N allantoin from biological samples was 97–101% (Table 2). The recovery of essentially all the labelled allantoin confirms that the reliability of the protein precipitation method as well as showing that there were minimal matrix effects. This was confirmed by the recovery of unlabelled allantoin that was added to biological samples, which was between 96 and 100% in plasma and 98–101% in urine samples (Table 2). When urate was added to plasma there was no increase in the detectable concentration of allantoin (data not shown). This indicates that urate was not oxidised to allantoin during the processing and analysis.

3.3. Analysis of clinical samples

Allantoin could be detected readily in plasma, urine, and synovial fluid. The median concentration of allantoin in the plasma of healthy volunteers was 2.0 μM (IQR 1.4–3.6 μM, $n=35$). These levels are similar to those reported by Gruber and colleagues [17,21] and Iwasaki et al. [22]. They are considerably lower than those reported by those using solid phase extraction and prolonged exposure of urate to alkaline conditions [8,16,23].

The median concentration of allantoin in the plasma of patients with rheumatoid arthritis was 3.7 μM (IQR 3.0–5.6 μM, $n=43$) while that in synovial fluid from patients with gout was 3.3 μM (IQR 2.8–5.8 μM, $n=10$). Both of these concentrations were significantly higher than that found in the plasma of healthy controls ($p < 0.001$; ANOVA on ranks). The higher level in synovial fluid may reflect the oxidative stress associated in inflammation [24] and the potential of myeloperoxidase to oxidise urate to allantoin [5]. These finding warrants further investigation. Previously, the concentration of allantoin in synovial fluid from patients with rheumatoid arthritis was reported to be 20.9 $\pm$ 7.3 μM compared to 18.6 $\pm$ 3.8 μM in serum of healthy controls [15]. The much higher values in the previous study most likely reflect artefactual oxidation of urate during derivatisation. Allantoin was easily detected in urine of healthy subjects but its concentration varied substantially (mean 28.76 μM; range 3.23–58.9 μM; $n=18$). There was both inter and intra day variation for different urine samples taken from the same donor. This demonstrates the need for a marker such as creatinine to standardise the concentration of the urine [25–28].

This HILIC–MS/MS method enables allantoin to be readily analysed in various biological matrices without any requirement for solid phase extraction or derivatisation. In addition there is at least a 10-fold improvement in previously reported detection limits [22,29] and accurate quantification is achieved by using a stable isotope of allantoin as an internal standard. Due to the high sensitivity and specificity of this method, it is possible to use as little as 10 μL of sample. Using HILIC also avoids the 2 h derivatisation step that the newer GC/MS method entails. The throughput of this method has the potential to be increased using ultra high performance liquid chromatography to shorten runs, as has recently been published [29]. The method also has the required sensitivity to detect

differences between allantoin levels of health controls and individuals with inflammatory pathologies associated with oxidative stress.

4. Conclusion

We have demonstrated that detection of allantoin using HILIC with LC/MS/MS is highly sensitive and specific. Also, this method is comparable to the newer GC/MS method [17] for determination of allantoin in clinical fluids. The main advantage of our current method is the ease of sample preparation because it does not require a long derivatisation step. It also has the potential to simultaneously monitor other metabolites of urate, such as triuret and 6-aminouracil [30]. This method has considerable potential for measuring allantoin as a biomarker of oxidative stress particularly in diseases associated with inflammation where urate will be oxidised to allantoin by myeloperoxidase [5].

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