

Determination of ^{15}N Isotopic Enrichment and Concentrations of Allantoin and Uric Acid in Urine by Gas Chromatography/Mass Spectrometry

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A method for the determination of ^{15}N enrichment and concentration of allantoin and uric acid simultaneously in urine using gas chromatography/mass spectrometry (GC/MS) is described. The urine samples contained $[1,3-^{15}\text{N}_2]$ uric acid and its oxidation product allantoin. The uric acid and allantoin were isolated using an AG1-X8 (Cl^- form) anion-exchange column and heated with a mixture containing 1:1 dimethylformamide and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA). The *tert*-butyldimethylsilyl (TBDMS) derivatives of allantoin and uric acid formed were injected into a gas chromatograph interfaced with a mass spectrometer operated under electron impact ionization conditions. Isotope ratio measurements were made from the abundance of the $\text{M}-57$ ions at m/z 398, 399 and 400 for allantoin and at m/z 567 and 569 for uric acid. $^{15}\text{N}_2$ allantoin (99 at.%) was produced from $[1,3-^{15}\text{N}_2]$ uric acid by treatment with uricase and used as a standard. Quantitation of allantoin and uric acid was based on isotopic dilution by spiking the urine sample with known quantities of 99 at.% $[^{15}\text{N}]$ uric acid and allantoin internal standards. The observed isotope ratio measurements from the prepared standards matched the theoretical values. Coefficients of variation in measurements of isotope ratio and concentration were 0.2 and 0.5%, respectively. The method was applied in a study to measure the urinary recovery of $[1,3-^{15}\text{N}_2]$ uric acid continuously infused for 8–10 h into the blood of four sheep each on two occasions. Within 24 h, $65.9 \pm 9.1\%$ of the tracer was excreted in the urine unchanged. Little was converted into allantoin ($\sim 7\%$ of the dose). The total recovery (5 days) of the infused tracer averaged $69.5 \pm 7.6\%$ as uric acid and $76.8 \pm 9.3\%$ as the sum of uric acid and allantoin. Uricase activities in plasma, liver and kidney of sheep were also measured using $[1,3-^{15}\text{N}_2]$ uric acid as a substrate. Uricase activity was estimated to be 0.6 mU g^{-1} wet tissue in the liver and there appeared to be none in plasma and kidney. The low uricase activities in sheep tissues appeared to explain the limited conversion of the intravenously administered $[^{15}\text{N}]$ uric acid to allantoin but did not explain the large quantities of allantoin excreted in urine (8.96 ± 0.86 and $1.36 \pm 0.25 \text{ mmol d}^{-1}$ for allantoin and uric acid, respectively). The GC/MS method for the determination of ^{15}N enrichment and concentration of allantoin and uric acid in urine is accurate and precise and provides a useful tool for studies on uric acid and allantoin metabolism.

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INTRODUCTION

Uric acid and allantoin are catabolism products of purines. Methods for the determination of ^{15}N isotopic enrichment and quantitation of uric acid based on gas chromatography/mass spectrometry (GC/MS) have been reported.^{1–4} However, there has been no report of the determination of allantoin based on GC or MS, although a range of methods based on high-performance liquid chromatography, colorimetry and titration are available for cosmetic, pharmaceutical and biological samples (see review by Chen *et al.*⁵).

Ruminants excrete large quantities of uric acid and allantoin in the urine since they absorb substantial amounts of purines associated with microbial cells syn-

thesized in the forestomach. The daily output of allantoin and uric acid is directly correlated with the uptake of exogenous purines^{6,7} and therefore provides an index of intestinal flow of microbial biomass in ruminants. Uric acid and allantoin produced in the body enter the circulation and are mainly excreted in the urine. A small fraction can be disposed of by a non-renal route, e.g. by secretion to the gut via saliva.⁸ In this work, we were interested in determining the proportion of uric acid and allantoin excreted in the urine of sheep after an intravenous infusion of $[1,3-^{15}\text{N}_2]$ uric acid as a tracer. It was expected that the tracer would appear in the urine unchanged and as its metabolite allantoin. In order to provide additional information as to the fate of uric acid, we also examined the activities of uricase (EC 1.7.3.3), which catalyses the conversion of uric acid to allantoin, in plasma, liver and kidney of sheep using $[1,3-^{15}\text{N}_2]$ uric acid as the substrate. This paper reports a method to measure the ^{15}N isotopic ratios and concentration, of uric acid and allantoin simultaneously in urine by GC/MS and its applications to the animal

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studies. This is the first report of a method for allantoin determination based on GC/MS.

EXPERIMENTAL

Analytical procedures

Reagents. Dimethylformamide (DMF) and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) were purchased from Pierce (Chester, UK). Ammonia solution, allantoin, uric acid and uricase (Sigma Cat. No. U-3377, 6.3 U mg⁻¹ protein) were obtained from Sigma (Poole, UK). [1,3-¹⁵N₂]Uric acid (99 at.%) was acquired from C K Gas Products (Miamisburg, OH, USA) and AG1-X8 resin (100–200 mesh) from Bio-Rad (Hemel Hempstead, UK). [8-¹⁴C]Uric acid and [4,5-¹⁴C₂]allantoin were purchased from Amersham International (Amersham, UK).

Standards. Standards for determination of isotope ratio of uric acid. Two aqueous standard solutions were prepared: one contained natural uric acid and the other [1,3-¹⁵N₂]uric acid, each at 300 µmol l⁻¹. To improve the solubility of uric acid, NaOH (0.6 M) was added to give a final NaOH concentration of 0.25 mM. These two stock solutions were mixed in different proportions to produce six working standard solutions. The range of theoretical ¹⁵N enrichments covered by the standards was 0–18 mol% excess (MPE), which was calculated as follows:

$$\text{MPE} = \frac{R}{1 + R} \times 100\% \quad (1)$$

where *R* is the molar ratio of [¹⁵N]uric acid to natural [¹⁴N]uric acid.

Standards for determination of isotope ratio of allantoin. Since [¹⁵N]allantoin was not commercially available, allantoin enrichment standards were prepared by treating the enriched uric acid working standards with uricase. The procedure was as follows: 3 ml of each of the [¹⁵N]uric acid working standards was mixed with 200 µl of uricase solution (0.15 U ml⁻¹ in water). The pH of the uric acid solutions was between 8.5 and 9.5. The mixture was incubated at 37 °C for 45 min. The absorbance at 293 nm at the end of incubation was close to zero.

Preparation of 99 at.% [¹⁵N₂]allantoin internal standard. A 250 ml volume of a solution containing 300 µmol l⁻¹ 99 at.% [1,3-¹⁵N₂]uric acid was prepared in 0.25 mM NaOH. The solution was then split into several 30 ml aliquots. The uricase solution (0.15 U ml⁻¹ in water) was added to give 0.01 U uricase per ml of the uric acid substrate solution. The mixture was then incubated at 37 °C for up to 3 h. The disappearance of absorbance at 293 nm at the end of incubation confirmed complete disappearance of uric acid. All aliquots were then pooled and concentrated to about 30 ml by rotary evaporation at 60 °C. The resultant solution was filtered through a 0.20 µm syringe filter and made up to 50 ml with distilled water. GC/MS analysis confirmed that there was no uric acid present

and the allantoin was 99 at.% enriched. The product was a mixture of [1,3-¹⁵N₂]allantoin and [7,9-¹⁵N₂]allantoin (see discussion). For simplicity, the numbering of the positions of ¹⁵N in allantoin are thereafter omitted. The concentration of the [¹⁵N₂]allantoin was determined by dilution with known amounts of natural allantoin. The [¹⁵N₂]allantoin tracer solution prepared in this work was 1.37 mmol l⁻¹.

Isolation and derivatization. Urine samples and standard solutions which contained mineral salts were desalted as follows before derivatization. The sample or standard solution (1–6 ml) was mixed with 0.5 ml of 6 M ammonia solution and applied to a 3.5 cm × 10 mm i.d. column packed with an anion-exchange resin (AG1-X8, 100–200 mesh, chloride form). The column was washed with 4 ml of water and allantoin and uric acid were eluted with 4 ml 0.1 M HCl. A 500 µl volume of the eluate, which contained ~30 nmol uric acid and 150 nmol allantoin, was transferred to a V-vial and dried at 90 °C under nitrogen. In the case of standard solutions that had not been previously passed through the AG1-X8 column, 100 µl were transferred into V-vials. The *tert*-butyldimethylsilyl (TBDMS) derivatives of allantoin and uric acid were formed by reacting with 80 µl of DMF–MTBSTFA (1:1) at 130 °C for 20 min. The reaction mixture was then analysed by GC/MS.

GC/MS instrumentation and conditions. The instrumentation consisted of an HP 5890 gas chromatograph coupled to an HP 5989A quadrupole mass spectrometer (Hewlett-Packard, Manchester, UK). The gas chromatograph was equipped with a 30 m × 0.25 mm i.d. (0.25 µm film thickness) SE-30 CB capillary column (Alltech, Carnforth, UK). Injections (1 µl) were made in the split mode with the split ratio set to 40:1. Helium was used as the carrier gas at a head pressure of 76 kPa. The injector temperature was 280 °C and the column temperature programme was from 150 to 270 °C at 20 °C min⁻¹ for 9 min. The GC/MS interface temperature was 260 °C.

The mass spectrometer was operated under electron impact ionization conditions with the following source parameters: electron energy, 70 eV; emission current, 300 µA; and source temperature, 200 °C. The *M* – 57 ions at *m/z* 398, 399 and 400 for allantoin and *m/z* 567 and 569 for uric acid were monitored under selective ion recording conditions with a 30 ms dwell time on each ion. The detector voltage was set at 1150 V for allantoin and 1250 V for uric acid.

Measurement of the ¹⁵N enrichments of uric acid and allantoin. The MPE was calculated based on Eqn (1), *R* being calculated as follows: for uric acid:

$$R = R_i - R_0 \quad (2)$$

for allantoin:

$$R = (R_{i1} - R_{01})(1 - R_{01}) + (R_{i2} - R_{02}) \quad (3)$$

Both equations were derived from study of the ion distributions of the analyte concerned. For uric acid, *R* corresponds to the molar ratio of [¹⁵N]uric acid to [¹⁴N]uric acid. *R_i* and *R₀* are the peak area ratios of *m/z* 569/567 of the enriched and natural uric acid, respectively. [¹⁵N]Allantoin formed from [1,3-

$^{15}\text{N}_2$]uric acid gave rise to not only a doubly labelled ion but also a singly labelled ion. R in Eqn (3) corresponds to the molar concentration ratio of the sum of singly and doubly labelled ^{15}N]allantoin relative to ^{14}N]allantoin. R_{01} and R_{02} are the m/z 399/398 and m/z 400/398 peak area ratios of natural allantoin, respectively. R_{i1} and R_{i2} are the m/z 399/398 and m/z 400/398 peak area ratios of enriched allantoin, respectively.

Quantitation of uric acid and allantoin. Quantitation was based on isotope dilution, using 99 at.% ^{15}N internal standards. The internal standard solutions used were $300\ \mu\text{mol l}^{-1}$ $[1,3-^{15}\text{N}_2]$ uric acid and $1.37\ \text{mmol l}^{-1}$ $^{15}\text{N}_2$]allantoin solution. Because the internal standards were the same isotopes as those present in the urine following administration of ^{15}N]uric acid, the values of isotope ratios measured after addition of internal standards for the purpose of quantitation must be corrected for the isotope ratios of the same sample measured without addition of internal standards.

For quantitation of uric acid and allantoin, working standards were prepared by spiking five 1 ml aliquots of a urine sample with 1 ml of each of the internal standards and varying amounts of natural uric acid (0, 0.25, 0.50, 0.75 and 1.00 ml at $300\ \mu\text{mol l}^{-1}$) and natural allantoin (0, 0.5, 1.0, 1.5 and 2.0 ml at $1.27\ \text{mmol l}^{-1}$). The standards were taken through the analytical procedure and analysed by GC/MS. The concentration ranges covered by the standards were $0\text{--}300\ \mu\text{mol l}^{-1}$ for uric acid and $0\text{--}2.54\ \text{mmol l}^{-1}$ for allantoin. Urine samples were diluted so that the concentrations were within the range. Two 1 ml aliquots were taken from each urine sample, one of which was spiked with 1 ml of each of the internal standards. The two aliquots were then subjected to the same procedure as the standards.

Calculation was made as follows. From the working standards, a linear regression between the measured $^{14}\text{N}:^{15}\text{N}$ molar concentration ratio (R') for uric acid or allantoin and the actual $^{14}\text{N}:^{15}\text{N}$ molar concentration ratios was established and the regression coefficient (b) thus determined. R' was the reciprocal of R (i.e. $R' = 1/R$) calculated according to Eqns (2) and (3) for uric acid and allantoin, respectively. The molar concentration of ^{14}N]uric acid or allantoin in the sample was calculated as $Y = I/[(R_a - R_b) \times b]$, where I is the concentration of the internal standard and R_a and R_b are the measured R values for each urine sample with and without addition of internal standard, respectively. In urine samples containing only natural uric acid and allantoin, R_b was zero. However, in urine samples containing ^{15}N]uric acid or allantoin, R_b was >0 but $<R_a$. The concentration of the 99 at.% ^{15}N]uric acid or allantoin in these samples was calculated as YR_b .

Animal experimentation

Intravenous infusion of $[1,3-^{15}\text{N}_2]$ uric acid to sheep. Four sheep (Suffolk \times Greyface, male, about 8 months old, average body mass 40 kg) were fed with $1.0\ \text{kg d}^{-1}$ of a mixed ration containing 50% hay, 30% barley, 10% molasses, 9% fishmeal and 1% mineral and vitamin mixture. The feed was given in two equal meals at 0800 and 1630 hours. The animals were introduced to the

diet and level of feeding 10 days before being moved to metabolism cages. Complete collection of urine and faeces was made daily for 7 days. The animals were then fitted with a jugular vein catheter and infused with sterilized saline containing $0.44\ \text{mmol l}^{-1}$ of 99 at.% $[1,3-^{15}\text{N}_2]$ uric acid using a Harvard syringe pump (Harvard Apparatus, South Natick, MA, USA) for 8–10 h at a rate of $57\ \text{ml h}^{-1}$. Complete collection of urine was continued for the next 7 days from the start of tracer infusion. After a 5 week interval, the tracer infusion was repeated with a lower concentration of $[1,3-^{15}\text{N}_2]$ uric acid ($0.22\ \text{mmol l}^{-1}$). The dose of $[1,3-^{15}\text{N}_2]$ uric acid averaged 225 and $119\ \mu\text{mol}$ for the two periods respectively.

Urine was collected daily in 100 ml of 1 M H_2SO_4 solution so that the pH was about 2–3. The urine ($0.6\text{--}1.5\ \text{l d}^{-1}$) was then diluted to 3 l with water. In the second infusion period, the urine was collected in 8 h fractions on the first day of infusion, but daily on other days. Subsamples of urine were stored at -20°C until analysis.

Measurement of uricase activity in liver and kidney extracts and in plasma. A liver and a kidney were acquired each from one sheep immediately after slaughter. The organs were kept in ice and treated within 3 h using the following procedure adapted from that of Furth-Walker and Amy⁹. The organs were washed with cold 0.15 M KCl and wiped dry with paper. About 75 g of the tissue of each organ were weighed and homogenized with $\sim 200\ \text{ml}$ of 0.5 mM (EDTA) in 0.05 M KH_2PO_4 buffer (pH 7.5) and centrifuged at $40\,000\ g$ for 20 min at 4°C . The supernatants were transferred to dialysis bags (inflated diameter 19.0 mm, molecular mass cut-off 12–14 kDa) and dialysed against the same EDTA- KH_2PO_4 buffer at 4°C for 20 h. The contents of the dialysis bag were again centrifuged at $40\,000\ g$ for 20 min at 4°C . The supernatant was made up to a volume with the EDTA- KH_2PO_4 buffer such that 1 g of wet tissue gave 3 ml of extract solution. About 20 ml of blood were collected from a sheep into heparinized tubes, centrifuged at $2500\ g$ for 15 min and the plasma was used for enzyme assay within 1 h.

The uricase activities in the sheep tissue extracts and plasma were examined using the following procedure. For each material in question, two sets of seven tubes were prepared. All tubes contained 1 ml of $300\ \mu\text{mol l}^{-1}$ 99 at.% $[1,3-^{15}\text{N}_2]$ uric acid, 1 ml of $1.37\ \text{mmol l}^{-1}$ natural allantoin, 1 ml of 0.1 M sodium acetate buffer (pH 8.6) and 1 ml of the tissue extracts or plasma. The exact amounts of ^{15}N]uric acid and natural allantoin added were recorded by masses. In one set, 200 μl of uricase solution ($0.15\ \text{U ml}^{-1}$ in sodium acetate buffer) giving 30 mU of uricase were added. Into one tube from each set, 0.4 ml of 3.5 M perchloric acid (PCA) was added immediately following the addition of the tissue extracts or plasma. This precipitated the protein and thus stopped the enzyme reaction. The other six tubes from each set were incubated at 37°C for 30, 60, 90, 120, 150 and 180 min, respectively. The reaction was stopped by addition of 0.4 ml of the PCA solution. All tubes were then centrifuged at $40\,000\ g$ at 4°C for 20 min. The supernatants were neutralized with 1.25–1.35 ml of 2 M KOH to remove excess PCA

and centrifuged again at 40 000 *g* for 20 min. The supernatants were then taken through the analytical procedure and analysed by GC/MS. An increase in the ^{15}N enrichments of allantoin in the products would indicate the presence of uricase. One unit of uricase activity was defined as 1 μmol allantoin produced per minute at 37°C with excess substrate.

RESULTS AND DISCUSSION

Sample preparation

The presence of salts (including potassium phosphate and sodium acetate) significantly blocked the derivatization of both allantoin and uric acid. The elution through the anion-exchange column (AG1-X8) was found to be an effective method of desalting. The behaviour of uric acid and allantoin on passage through the column was studied using $[8-^{14}\text{C}]\text{uric acid}$ and $[4,5-^{14}\text{C}_2]\text{allantoin}$ as tracers. The tracers were added to the samples and carried through the described protocol. The results are shown in Table 1. Both uric acid and allantoin (in their negatively charged form at pH 11) were completely (99%) bound to the column irrespective of the volume of sample loaded to the column. When eluted with 4 ml of 0.1 M HCl, 95–98% of the allantoin, but only 65–75% uric acid, were recovered. Complete recovery of uric acid required a larger elution volume (10 ml). However, incomplete recovery did not affect the accuracy of isotopic ratio determination and quantitation based on isotope dilution.

Mass spectrum and isotopic ratio measurements

The EI mass spectra of the TBDMS derivatives of natural and enriched uric acid and allantoin are shown in Figs 1 and 2. When $[1,3-^{15}\text{N}_2]\text{uric acid}$ is converted into allantoin by uricase *in vitro* or *in vivo*, the product is a mixture of $[1,3-^{15}\text{N}_2]\text{allantoin}$ and $[7,9-^{15}\text{N}_2]\text{allantoin}$. This is due to the fact that the intermediate compound immediately leading to allantoin can cleave randomly either between positions 1 and 5 or between positions 7 and 9. There is also an indication that this reaction is reversible,¹⁰ thus making possible the co-existence of $[1,3-^{15}\text{N}_2]\text{allantoin}$ and $[7,9-^{15}\text{N}_2]\text{allantoin}$ in equal proportions. During derivatization of allantoin, the terminal $\text{H}_2\text{N}'\text{CO}'$ group is lost, and therefore when $[1,3-^{15}\text{N}_2]\text{allantoin}$ is derivatized, a singly labelled derivative is formed, but when $[7,9-^{15}\text{N}_2]\text{allantoin}$ is derivatized, a doubly labelled derivative is formed, as shown in Fig. 3. From GC/MS

analysis, almost equal amounts of singly ($50.5 \pm 0.1\%$) and doubly ($49.5 \pm 0.1\%$) labelled TBDMS-allantoin were produced.

The mass chromatograms of the TBDMS derivatives of uric acid (*m/z* 567 and 569) and allantoin (*m/z* 398, 399 and 400) isolated from a sheep urine sample are shown in Fig. 4. The chromatograms show good chromatographic peak shapes and no interfering ions.

Within the range of ^{15}N enrichments covered in the uric acid standards, the MPE of uric acid (E_U , %) measurements were in good agreement with the theoretical values (E , %): $E_U = -0.0622 (\pm 0.043) + 0.969 (\pm 0.003)E$ ($r^2 = 1.000$, $P < 0.001$, $n = 6$). With the allantoin standards which were enzymatically derived from enriched uric acid, the measured MPE (E_A , %) also closely matched those of its precursor uric acid (E_U , %): $E_A = -0.001 (\pm 0.041) + 1.016 (\pm 0.003)E_U$ ($r^2 = 1.000$, $P < 0.001$, $n = 6$).

The coefficient of variation for repeated measurements of ^{15}N enrichment for both uric acid and allantoin was $<0.2\%$. The results clearly indicate that the procedure used gave accurate and precise measurements of the ^{15}N enrichment of uric acid and allantoin. TBDMS derivatives of uric acid and allantoin did not have any column memory effect in enrichment measurements.

Quantitation of allantoin and uric acid

There was a linear relationship ($r^2 = 0.9999$) between the concentration of natural uric acid corrected for $[^{15}\text{N}]\text{uric acid}$ internal standard and the peak area ratios of *m/z* 567/569 and between the concentration of natural allantoin corrected for ^{15}N allantoin internal standard and the peak area ratios of *m/z* 398/399. Similarly, the measured $^{14}\text{N}:^{15}\text{N}$ molar concentration ratio in the working standard mixtures was linearly correlated ($r^2 = 0.9999$) with the actual molar concentration ratio of $^{14}\text{N}:^{15}\text{N}$ for both uric acid and allantoin. The regression coefficients of these standard curves were used for the quantitation of uric acid and allantoin in the samples. The precision of quantitation was examined by splitting urine samples into several aliquots, which were analysed for $[^{14}\text{N}]\text{uric acid}$ and allantoin concentrations according to the protocol. The coefficients of variation were $<0.5\%$ (Table 2).

Quantitation by isotope dilution is generally regarded as a candidate definitive method,^{2–4} since the method is less subject to interference than methods based on chromatography or colorimetry. Accurate quantitation can be achieved if isotopic enrichment measurements are

Table 1 Behaviour of uric acid and allantoin in urine when applied to a 3.5 cm \times 10 mm i.d. AG1-X8 column

Tracer	Volume loaded (ml)	Binding efficiency ^a	Recovery in first 4 ml of HCl	Recovery in 8 ml of HCl
^{14}C Uric acid	1.5	0.99 ± 0.001	0.75 ± 0.019	0.92 ± 0.014
^{14}C Uric acid	6.5	0.99 ± 0.001	0.75 ± 0.019	0.92 ± 0.025
^{14}C Allantoin	1.5	0.99 ± 0.002	0.97 ± 0.008	0.98 ± 0.006
^{14}C Allantoin	6.5	1.00 ± 0.000	0.93 ± 0.051	0.95 ± 0.052

^a Binding efficiency = radioactivity applied to the column minus the sum of radioactivity collected in the sample and rinse fractions.

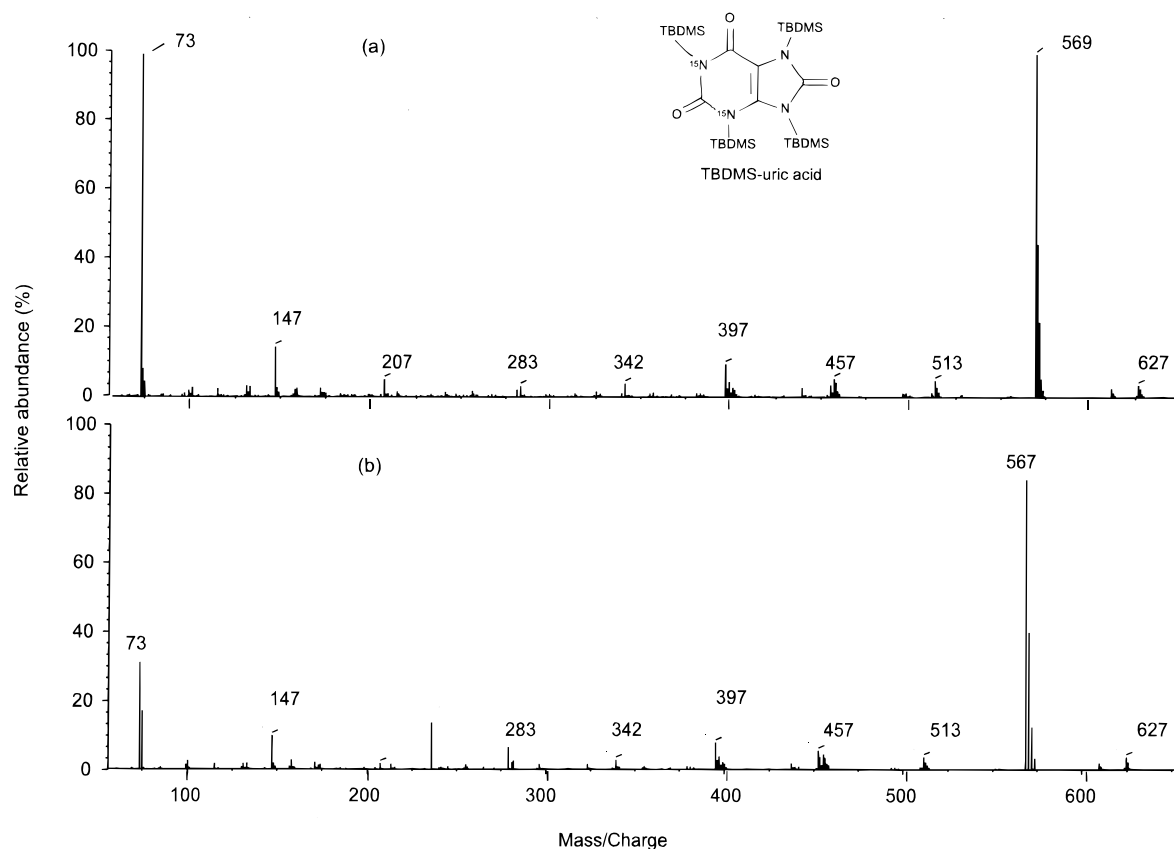


Figure 1. EI spectrum of TBDMS derivative of uric acid. The $M - 57$ ions at m/z 567 and 569 were monitored. (a) 99 at.% $[1,3-^{15}\text{N}_2]$ uric acid; (b) natural uric acid.

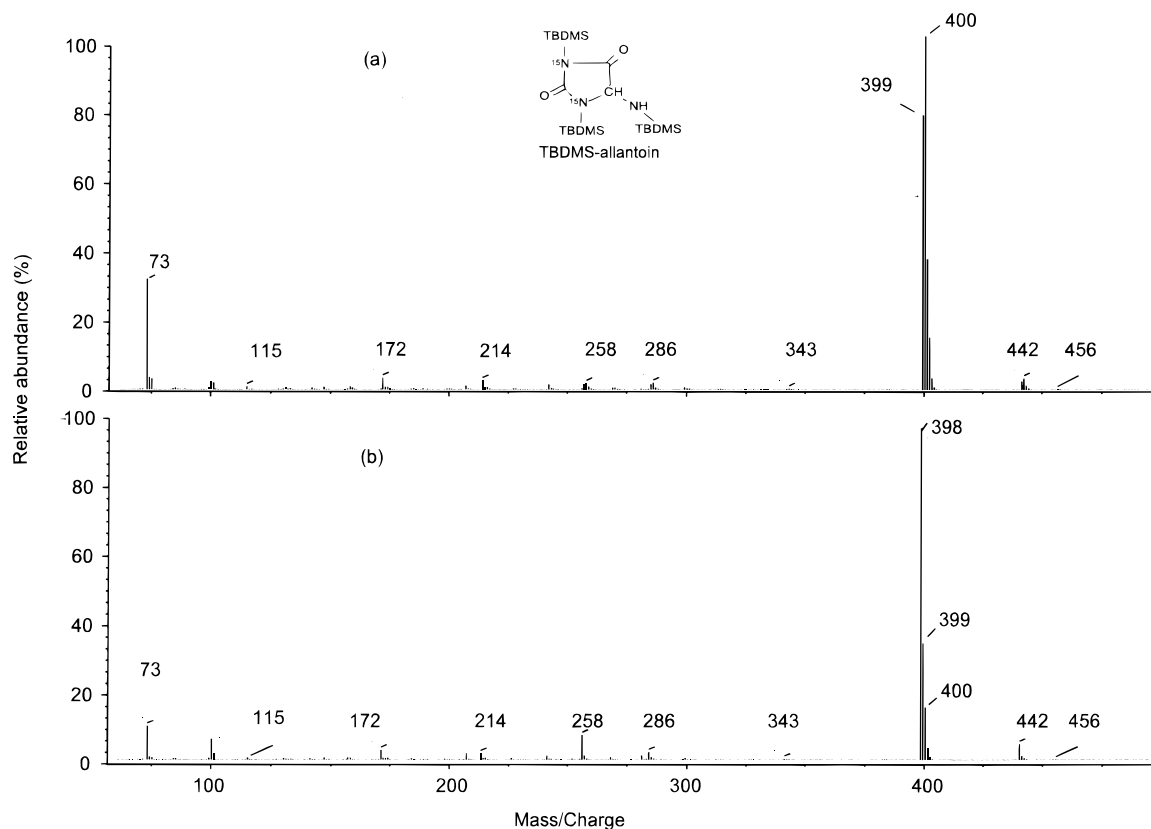


Figure 2. EI mass spectrum of TBDMS derivative of allantoin. The $M - 57$ ions at m/z 398, 399 and 400 were monitored. (a) 99 at.% $[^{15}\text{N}_2]$ allantoin; (b) natural allantoin.

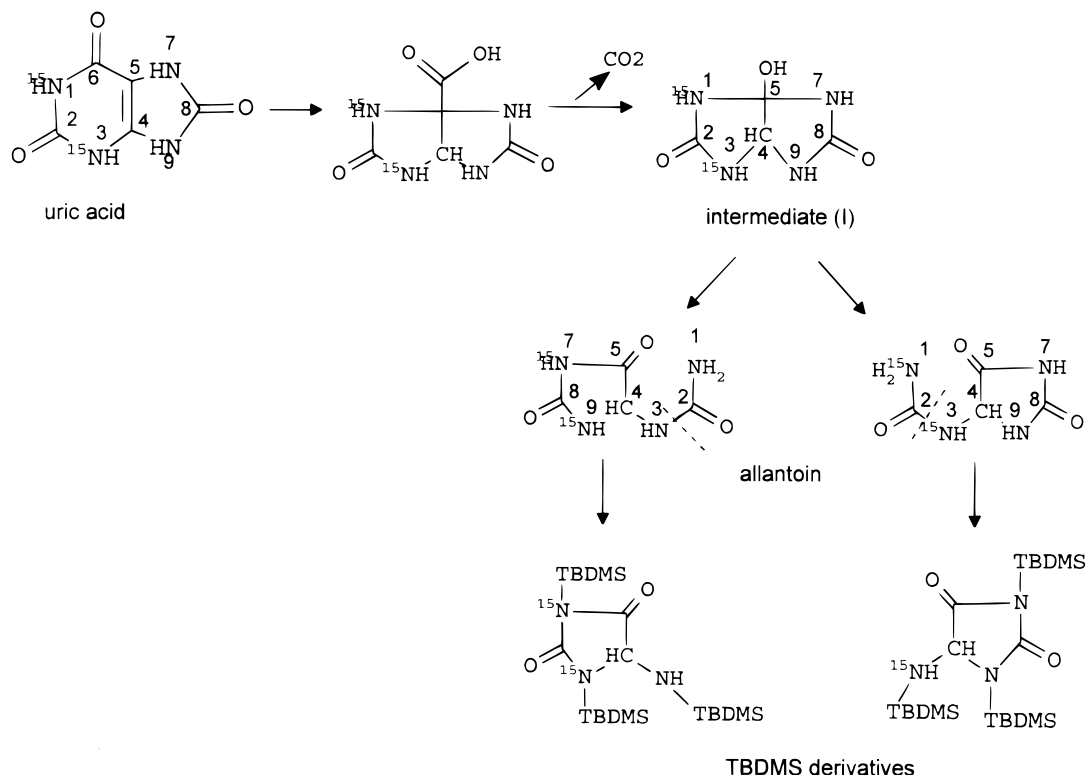


Figure 3. Formation of the TBDMS derivatives of singly and doubly labelled [^{15}N]allantoin.

accurate and precise. In the present procedure, the standards were prepared using urine as a medium in an effort to ensure that the calibration curve established was applicable to urine samples. Although the procedure was primarily developed for sheep urine, the approach should be applicable to other biological samples. Sheep urine contains high concentrations of uric acid and allantoin, and normally requires dilution by a factor of 4–5 so that the expected concentrations of uric acid and allantoin were similar to those of the ^{15}N -labelled internal standards added. We are currently investigating the use of this method for the analysis of

plasma samples of ruminants, which contain low concentrations of uric acid.

Isotopic ratio measurements in urine of sheep given i.v. infusion of [$1,3\text{-}^{15}\text{N}_2$]uric acid

Following intravenous infusion of [$1,3\text{-}^{15}\text{N}_2$]uric acid, the ^{15}N enrichment of uric acid in the urine increased on the first day to 14.1 ± 2.8 and $8.8 \pm 1.8\%$ for the two levels of tracer infusion. Within the day, the enrich-

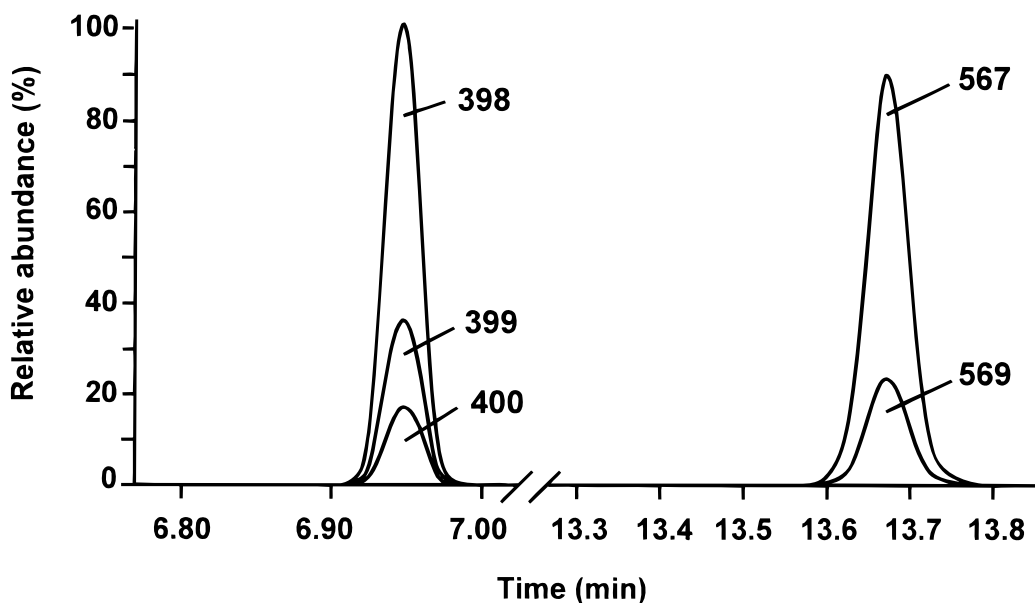


Figure 4. Selective ion recording traces of the TBDMS derivatives of uric acid (m/z 567 and 569) and allantoin (m/z 398, 399 and 400) isolated from a sheep urine sample.

Table 2. Reproducibility of measurement of uric acid and allantoin concentrations in sheep urine by isotope-dilution

Analyte	<i>n</i>	Mean \pm SD (mmol l ⁻¹)	CV (%)
Uric acid	5	0.277 \pm 0.0014	0.52
	5	0.252 \pm 0.0008	0.31
	mean		0.42
Allantoin	5	2.334 \pm 0.008	0.35
	3	3.758 \pm 0.016	0.42
	3	2.881 \pm 0.012	0.43
	3	4.223 \pm 0.027	0.65
	mean		0.46

ment was $9.0 \pm 2.3\%$ for the first 8 h and increased to $12.9 \pm 2.2\%$ for the second 8-h. Following the end of infusion, enrichment reduced to $4.0 \pm 1.5\%$ (the third 8 h). The enrichment reduced to $0.4 \pm 0.2\%$ on day 2 of infusion for both levels of tracer infusion. The data indicate that uric acid in the plasma of sheep had a small pool size and clearance was rapid.

The ¹⁵N enrichment of allantoin in the urine averaged 0.20 ± 0.13 and $0.09 \pm 0.08\%$ on the first day of infusion for the two levels of tracer dosed. From day 2 of infusion onward, the enrichment was below the detection limit. The extremely low enrichment in allantoin and high enrichment in uric acid were unexpected, since the dose of [1,3-¹⁵N₂]uric acid was intended to achieve equal enrichments in uric acid and allantoin taking into account the conversion of uric acid to allantoin. However, the results indicate that only a small fraction of the uric acid in the plasma was converted into allantoin.

Urinary recovery of [1,3-¹⁵N₂]uric acid

The cumulative outputs of the ¹⁵N tracers are shown in Table 3. It should be noted that the amounts of [¹⁵N]allantoin produced were approximates since the enrichment measurements were near the limit of detection. An average of $66 \pm 9.1\%$ of the infused [1,3-¹⁵N₂]uric acid was excreted unchanged within 24 h. The total recovery (5 days) of the tracer as uric acid averaged $69.5 \pm 7.6\%$. Taking into account the conversion to [¹⁵N]allantoin, the total recovery of the infused tracer was $76.8 \pm 9.3\%$. Assuming that allantoin and uric acid behave in a similar manner in renal excretion, the data indicate that an average of 77% of the uric acid entering the plasma is excreted in the urine and 23% is lost by a non-renal route. Judged from the relatively large standard deviation, there appeared to be a considerable variation between individuals in the renal/non-renal partitioning.

Uricase activity in plasma, liver and kidney extracts of sheep

Figure 5 shows the ¹⁵N enrichment of allantoin in the reaction mixture in which [¹⁵N]uric acid was incubated with liver and kidney extracts or plasma of sheep, with or without additional uricase. In the mixtures with added uricase (30 mU), the enrichment reached plateau by 30 min at 17.1 ± 0.05 , 17.9 ± 0.05 and $15.9 \pm 0.28\%$ for liver, kidney and plasma, respectively. All the uric acid disappeared, suggesting its complete conversion. It is calculated that up to 0.28 μ mol of [¹⁵N]allantoin was produced from the total of 0.29 μ mol of [¹⁵N]uric acid added. In the mixtures without added uricase, however, there was only a small elevation in the ¹⁵N enrichment of allantoin and uric acid was still present. In the mixtures treated with the liver extract, the ¹⁵N enrichment

Table 3. Recovery of [1,3-¹⁵N₂]uric acid intravenously infused into four sheep

Parameter	2655		2656		824		2654	
	1	2	1	2	1	2	1	2
Tracer dosed (μ mol)	202.1	122.1	201.5	120.6	249.5	115.4	246.6	116.0
[1,3- ¹⁵ N ₂]uric acid excreted each day as proportion of dose:								
Day 1	0.667	0.574	0.762	0.484	0.730	0.698	0.639	0.714
Day 2	0.027	0.017	0.034	0.111	0.008	0.007	0.012	0.019
Day 3	0.015	0.000	0.006	0.004	0.002	0.005	0.002	0.008
Cumulative excretion ¹⁵ N tracer (μ mol):								
[1,3- ¹⁵ N ₂]uric acid	144.7	72.2	163.7	71.8	184.5	81.9	161.6	86.1
[¹⁵ N ₂]-allantoin	2.8	3.2	18.9	9.6	19.9	14.9	31.1	4.1
Recovery ^a :								
Uric acid only	0.716	0.591	0.812	0.595	0.740	0.710	0.655	0.742
Uric acid + allantoin	0.730	0.618	0.906	0.674	0.819	0.839	0.781	0.778
Daily excretion of total uric acid and allantoin (mmol d ⁻¹) ^b :								
Uric acid	1.46 \pm 0.57	1.19 \pm 0.21	1.80 \pm 0.60	1.08 \pm 0.20	1.61 \pm 0.19	1.21 \pm 0.12	1.38 \pm 0.19	1.14 \pm 0.05
Allantoin	8.08 \pm 1.82	10.45 \pm 1.69	8.93 \pm 2.52	9.79 \pm 1.65	8.96 \pm 0.84	7.81 \pm 1.05	9.11 \pm 1.31	8.51 \pm 1.10

^a Cumulative excretion of ¹⁵N tracers over 5 days, expressed as a proportion of the dose.

^b Mean \pm SD of 5 days.

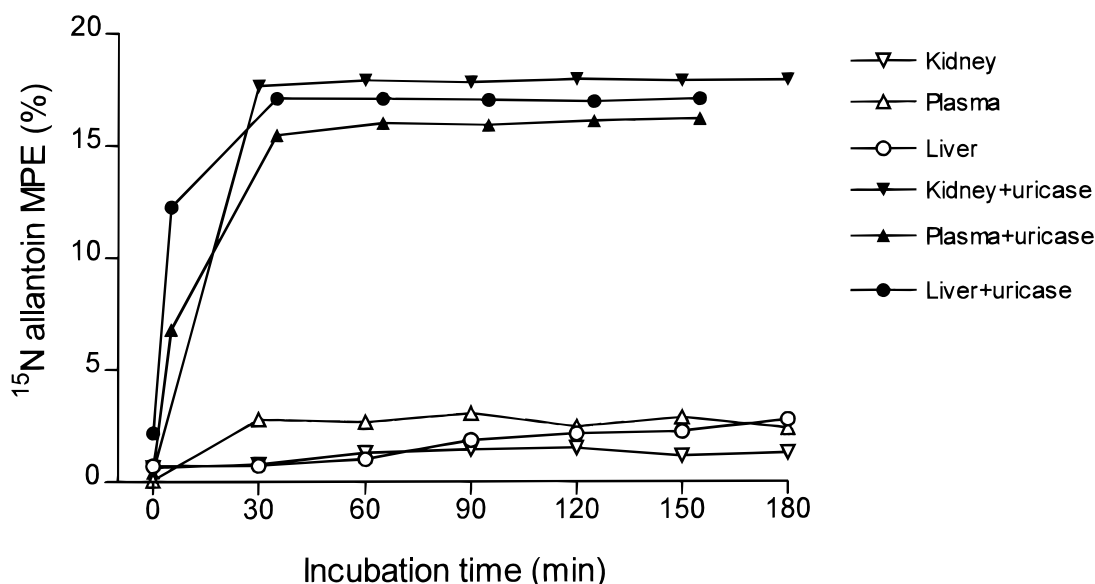


Figure 5. ^{15}N enrichment (MPE, %) in allantoine in the reaction mixture in which $[1,3-^{15}\text{N}_2]$ uric acid was incubated with liver and kidney extracts or plasma of sheep, with or without additional uricase (30 mU).

of allantoine increased linearly with time from 0.7 to 2.8% over 3 h, clearly indicating the presence of uricase. The uricase activity in the liver extract was estimated to be 0.6 mU g^{-1} wet tissue. In the mixtures treated with kidney extract and plasma, the ^{15}N enrichment of allantoine increased slightly within the first 30 min of incubation but thereafter remained unchanged (at 1.34 ± 0.18 and $2.68 \pm 0.29\%$, respectively) with time of incubation. It is not certain whether the initial increase in $[^{15}\text{N}]$ allantoine enrichment was truly due to uricase activity since the enrichment did not increase with time while the substrate was in excess.

There is little information as to the tissue distribution of uricase in sheep. In a study with rats, uricase was detected only in the liver and the contents of uricase in other tissues were estimated to be $<2\%$ of that in the liver.¹¹ The liver was probably also the only organ in sheep where a substantial amount of uricase is present. However, at 0.6 mU of uricase per gram of wet liver tissue, the amount of uricase present would only be sufficient to convert $0.86 \mu\text{mol}$ of uric acid to allantoine per gram of tissue within 24 h (0.7 mmol d^{-1} for a liver of 800 g). Although this may explain the limited conversion into allantoine of $[^{15}\text{N}]$ uric acid administered to

the blood, it does not explain the fact that large quantities of allantoine were excreted in the urine (8.96 ± 0.86 and $1.36 \pm 0.25 \text{ mmol d}^{-1}$ for allantoine and uric acid, respectively; see Table 3). The location and mechanisms of allantoine production need to be further examined.

The GC/MS method described permits the accurate and precise determination of ^{15}N enrichment and concentration of allantoine and uric acid in urine. Application of the method shows that in sheep, uric acid in the blood was converted into allantoine only to a limited extent, but was rapidly excreted in the urine. An average of 77% of uric acid entering blood was excreted in the urine. Sheep liver, kidney and plasma had low uricase activities. The GC/MS method should also provide a useful tool for studies of uric acid and allantoine metabolism in general.

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