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Measurement of allantoin in urine and plasma by high-performance liquid chromatography with pre-column derivatization

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ABSTRACT

A method is reported for determination of allantoin in urine and plasma based on high-performance liquid chromatography (HPLC) and pre-column derivatization. In the derivatization procedure, allantoin is converted to glyoxylic acid which forms a hydrazone with 2,4-dinitrophenylhydrazine. The hydrazone appears as *syn* and *anti* isomers at a constant ratio. These derivatives are separated by HPLC using a reversed-phase C_{18} column from hydrazones of other keto acids possibly present in urine and plasma and then monitored at 360 nm. All components were completely resolved in 15 min. Both the reagents and derivatization products are stable. Recovery of allantoin added to urine and plasma was 95 ± 3.7% (n = 45) and 100 ± 7.5% (n = 64), respectively. The lowest allantoin concentration that gave a reproducible integration was 5 μmol/l. The between-assay and within-day coefficients of variation were 2.8 and 0.6%, respectively.

INTRODUCTION

Allantoin is the end degradation product of purines in most mammals. Ruminant animals excrete substantial amount of allantoin in urine since they absorb large quantity of purines from microbial biomass produced during rumen fermentation. Measurement of urinary excretion of purine derivatives, including allantoin, uric acid, xanthine and hypoxanthine (allantoin accounts for about 85% of the total), provides an index of the amount of microbial biomass supplied to the animal. The use of this approach as a non-invasive method to estimate microbial protein supply has been investigated [1]. Methods for determination of allantoin based on HPLC have been reported [5–9]. However, some of the methods [5,8,9] employ long columns (or serial connection of two columns), and resolution is often not satisfactory. Moreover, allantoin is monitored at 195–218 nm which would be absorbed by many compounds in biological fluid samples. These features in combination affect the accuracy of quantification, particularly when the concentration of allantoin is low. It is desirable to convert allantoin to a derivative that can be readily resolved or monitored at a unique wavelength.

Determination of allantoin is widely carried out by the classical colorimetric method of Young and Conway [2]. The principle is to convert allantoin to glyoxylic acid (GLX) by sequential hydrolysis under alkaline and acidic conditions, and to react the GLX with phenylhydrazine to form a hydrazone which is measured colorimetrically. This procedure is, however, tedious and time-critical although it can be automated [3,4]. The GLX phenylhydrazone is not stable.

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Borchers [10] proposed that allantoin could be determined by colorimetric measurement of the 2,4-dinitrophenylhydrazone of GLX. Compared to the method of Young and Conway [2], this has the advantage of increased stability of the reagents used and the products of reaction, and the reactions do not require critical timing. However, the colorimetric assay for the 2,4-dinitrophenylhydrazone of GLX is not specific due to interference by 2,4-dinitrophenylhydrazones of other keto acids in samples of urine or plasma. Our studies (unpublished data) indicated that this method gave consistently higher values for urine than the Young and Conway method [2], and yielded exceedingly high readings for plasma samples. The problem of non-specificity of the colorimetric method can be overcome by the use of HPLC to separate the GLX hydrazone from interfering hydrazones prior to its measurement. Moreover, the 2,4-dinitrophenylhydrazone absorbs at 360 nm unlike other compounds orginally present in the biological fluid sample. Therefore, the combination of a pre-column derivatization and separation by HPLC could provide an alternative method for allantoin determination. This paper presents the results of our preliminary studies on the derivatization and chromatographic conditions of this method.

EXPERIMENTAL

Reagents

2,4-Dinitrophenylhydrazine (DNPH) (Sigma, UK) (1 g/l) was dissolved in 2 *M* HCl and filtered through Whatman No. 1 paper. Since the commercial product contains 30% water, the actual concentration was about 3.5 *M*. This solution was kept at 4°C. 0.6 *M* NaOH and pH indicator (0.04%, w/v, thymol blue) (Gurr's, London, UK) were dissolved in distilled, deionized water, and filtered through Whatman No. 1 paper. Allantoin standards (Sigma, UK) were 31, 63, 127, 190, 253 and 316 μmol/l in water.

Sample preparation

Urine was collected from animals into an appropriate amount of 1 *M* H_2SO_4 to decrease the pH to below 3. Urine samples were normally diluted by a factor of 5 prior to storage at −20°C. Before derivatization, the sample was further diluted so that the expected allantoin concentration was between 30 and 320 μmol/l.

Blood was collected into tubes containing heparin, and centrifuged at 1500 *g* for 20 min. The plasma was stored at −20°C. On the day of analysis, 2 ml of plasma were mixed with 2 ml of 10% (w/v) trichloroacetic acid as a deproteinizing agent and centrifuged at 35 000 *g* for 20 min. The supernatant was used for the derivatization procedure.

Derivatization procedure

A 500-μl sample (urine, plasma or standard) and 50 μl of the pH indicator were pipetted into a 1.5-ml Eppendorf centrifuge tube. The colour of the mixture was red if the sample was acidic (pH < 1.2). A 100-μl volume of 0.6 *M* NaOH solution was added. If the colour failed to change to blue (pH > 9.2 when blue), more NaOH solution was added in 50-μl increments (volume was recorded). After heating at 85°C (in a water bath) for 60 min, 200 μl of the DNPH solution were added. The colour of the mixture became orange–yellow. The heating was continued for 20 min. After cooling, the mixture was centrifuged at 35 000 *g* for 15 min and the supernatant injected into the HPLC system. If necessary, the mixture could be stored at −4°C for up to a week before injection. To provide calibration for the samples, a set of allantoin standards (31–316 μmol/l) were included in each batch of derivatization.

Chromatographic conditions

The HPLC equipment used consisted of a Waters 625 LC system (including a controller and a pump), a tunable absorbance detector (Waters Model 486), Waters 712 WISP autosampler, and computer data handling system (all equipment from Waters, Millipore, MA, USA). The analytical column was a Nova-Pak C_{18} (4 μm), 150 mm × 3.9 mm I.D. (Waters, Millipore). A guard column (Upchurch Scientific, UK) of 25 mm × 2 mm I.D. packed with reversed-phase C_{18} (30–40 μm) pellicular packing material was used.

Two solvents were used: (A) 15% acetonitrile in 0.01 *M* acetic acid (the latter was made up in distilled, deionized water, pH adjusted to 6.1 with ammonia solution); (B) acetonitrile. The reagents (glacial acetic acid and acetonitrile) were of HPLC grade (HiPerSolv, BDH, UK). Both solvents were filtered through a pore size 0.22-μm membrane filter (Anachem, UK). An initial degassing of the solvents was made by ultrasonication but on-line degassing was achieved by fluxing helium gas into the solvents at 30 ml/min. The gradient used was (% B): 0% at 0 min, 18% at 3 min (concavely increased from 0 min, line No. 7); 82% at 5 min (concavely increased from 3 min, line No. 7); 82% at 8 min; 0% at 9 min (linearly decreased from 8 min) and 0% at 15 min. Flow-rate was 1.0 ml/min. When B increased from 0 to 82%, the system pressure decreased from 11.72 to 6.21 MPa. Injection volume was 10 μl. Detector was set at UV 360 nm with an attenuation of 0.050 a.u.f.s. Total run time was 15 min. The separation was performed at room temperature.

RESULTS AND DISCUSSION

Chromatographic separation

Fig. 1 shows the chromatograms for a standard (allantoin in water), a urine sample, a plasma sample and a blank (water). GLX-DNPH appeared in the chromatograms as two peaks (re-

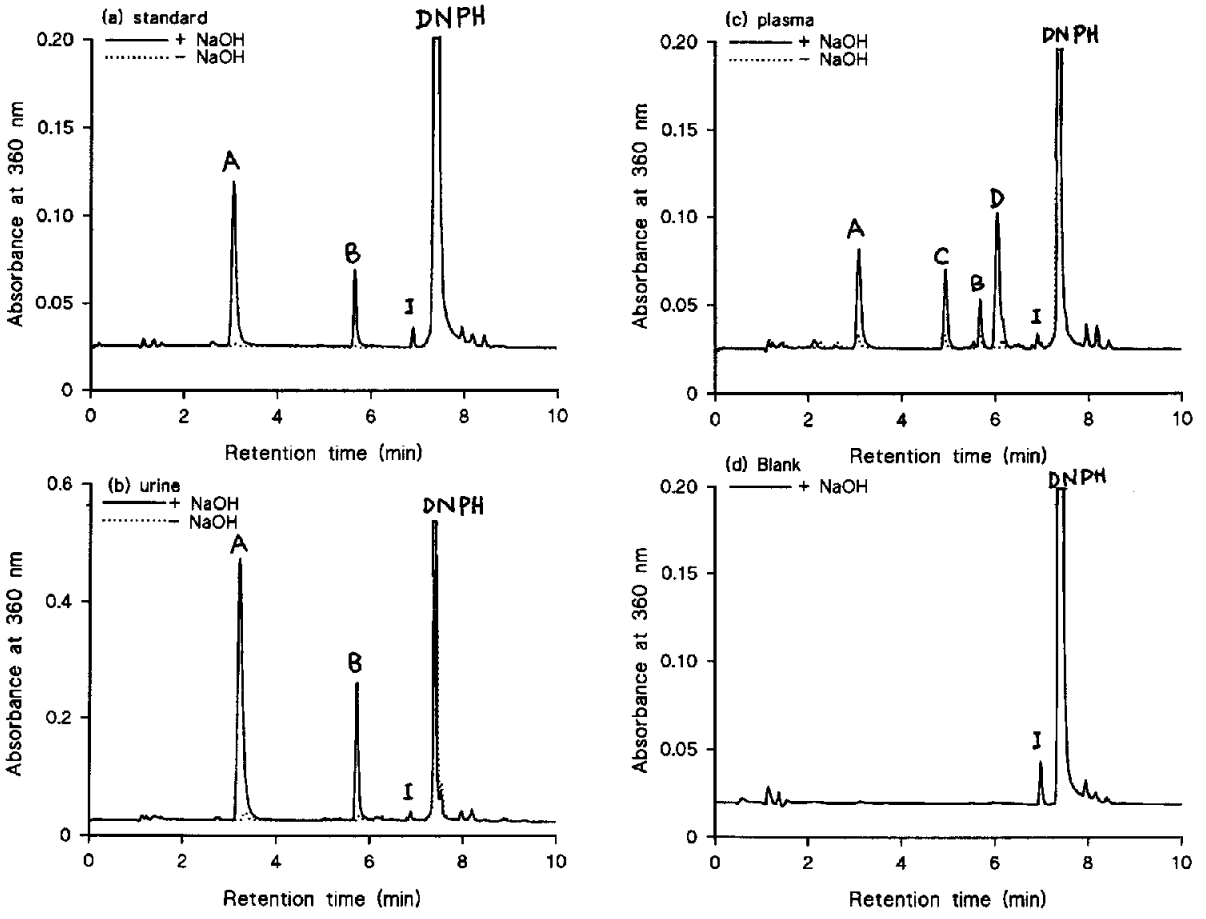

Fig. 1. Chromatograms for a standard (a), urine (b), plasma (c) and blank (d) with (———) or without (·····) hydrolysis of allantoin to glyoxylic acid under alkaline condition. The peaks and their retention times are: (A) allantoin-A at 2.96 min, (B) allantoin-B at 5.54 min, (I) thymol blue at 6.90 min and (DNPH) 2,4-dinitrophenylhydrazine at 7.34 min. C and D are unidentified compounds. Allantoin-A and -B are isomers of 2,4-dinitrophenylhydrazone of glyoxylic acid.

ferred to here as allantoin-A and allantoin-B), because GLX reacts with DNPH to yield *syn* and *anti* isomers [12,13]. These peaks had a retention time of (mean ± S.D. of 80 samples) 2.96 ± 0.027 and 5.54 ± 0.022 min, respectively, and were well separated from thymol blue (6.90 ± 0.017 min), DNPH (7.34 ± 0.015 min) and other unidentified complexes (peaks C and D in Fig. 1c). Peak identification was carried out by addition to the sample of the compound in question. As expected, allantoin-A and allantoin-B peaks were absent from the blank.

The ratio of allantoin-A/B peak area was constant at 0.77 (±0.007):0.23 (calculation based on 120 samples), irrespective of the concentration of allantoin in the sample. The ratio was identical for standards, urine and plasma samples. This observation is in agreement with the findings reported in the literature [12,13]. The peak response (peak area, y) of allantoin-A, -B or the sum of the two, was linearly related to allantoin concentration (x: μmol/l) over the concentration range tested: allantoin-A: $y = 5763\,x + 24394$ ($r = 0.999$, $p < 0.001$); allantoin-B: $y = 1707\,x + 6208$ ($r = 0.999$, $p < 0.001$); sum of A and B: $y = 7468\,x + 30394$ ($r = 0.999$, $p < 0.001$). All three parameters were therefore suitable for quantitative determination of concentration of the unknown, although the larger peak allantoin-A was preferred since accurate peak integration was readily achieved. However, the result calculated based on peak B, which should be close to that based on A, provided a quick indication of whether peak integration of either A or B had been accomplished correctly.

Pre-column derivatization

It is important to ensure an alkaline condition which is required for the hydrolysis of allantoin to allantoic acid. Also shown in Fig. 1a–c are chromatograms of the standard, urine and plasma samples which were processed using the derivatization procedure with the exception that NaOH was replaced with water. As expected, no allantoin-A nor allantoin-B peak was detected with the standard and urine sample and only very small allantoin-A and -B peaks were detected with the plasma sample. As a routine method of preservation, urine samples from our laboratory were acidified to a pH below 3, however, to a variable extent. The inclusion of a pH indicator (thymol blue) in the reaction mixture provided a visual aid to check whether sufficient alkalinity had been acquired after addition of NaOH.

In the development of the derivatization procedure, the effect of different extent of alkalinity on the hydrolysis of allantoin to allantoic acid was examined. In an experiment, four concentrations of NaOH (0.3, 0.6, 1.25 and 2.5 *M*) were applied to a set of standards (giving a NaOH concentration of 46–384 m*M*). Based on peak area of allantoin-A, no difference was observed between these concentrations. It appeared that complete hydrolysis of allantoin to allantoic acid was achieved under a weak alkaline (<46 m*M*) condition and was not affected by the extent of alkalinity. When the same experiment was applied to urine samples containing low concentrations of allantoin (thus analyzed undiluted) or plasma samples, NaOH at 2.5 *M* (final concentration 384 m*M*) gave a lower peak response than other concentrations, which showed no differences: the readings for 2.5 *M* NaOH were 93 ± 0.6% ($n = 4$) of the mean of other concentrations. It is possible that some unknown reaction was facilitated under strong alkalinity and reduced the GLX-DNPH formation. NaOH at 0.6 *M* was therefore adopted in the derivatization procedure and precaution was taken to avoid addition of excess NaOH.

During the method development, studies were also carried out to ensure that the amount of DNPH in the reaction mixture would not restrict GLX-DNPH formation. Different concentrations of DNPH (1, 2, 4 and 6 g/l in 2 *M* HCl) were applied to standards (31–316 μmol/l allantoin), urine and plasma. Results showed no differences in the peak area of allantoin-A and -B. In the finalized derivatization procedure, the amount of 1 g/l DNPH added was in excess of that required for reacting with GLX (final concentration 831 μmol/l). Within the range of allantoin concentration used (<320 μmol/l), a small reduction in DNPH concentration due to its reaction with other keto acids would not affect the extent of

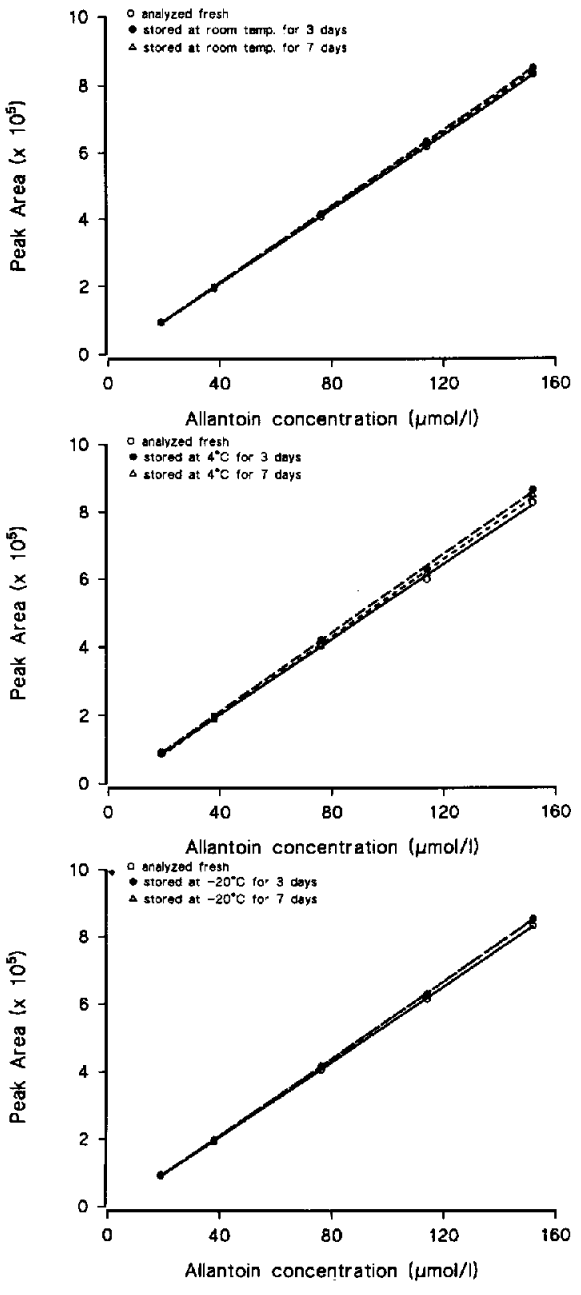

Fig. 2. Effect of method and length of storage on measurement of allantoin-A peak area. (Top) Stored at room temperature; (centre) stored at 4°C; (bottom) stored at −20°C. (○) Analyzed fresh; (●) stored for three days; (△) stored for seven days.

GLX-DNPH formation. Results of a time-course study showed that the formation of GLX-DNPH hydrazone was complete after 5 min at 85°C and the hydrazone did not disintegrate when heating was continued for 35 min. The GLX-DNPH *syn*/*anti* isomers are both stable. No reduction in peak area of allantoin-A or -B was observed when the processed samples were stored for seven days at room temperature, 4°C or −20°C (see Fig. 2).

TABLE I

RECOVERY OF ALLANTOIN ADDED TO SAMPLES OF URINE OR PLASMA

The determination of the concentration of unknown was based on allantoin-A peak.

Allantoin added (μmol/l)	*n*	Recovery[a] (mean ± S.D.) (%)
Urine		
31.6	15	96.2 ± 2.7
94.9	15	92.0 ± 4.7
158.2	15	95.7 ± 1.5
Pooled data	45	94.6 ± 3.7
Plasma		
31.6	9	103.4 ± 12.7
38.0	11	103.5 ± 7.2
75.9	3	101.6 ± 2.1
94.9	10	95.7 ± 7.2
113.9	12	99.7 ± 4.0
158.2	10	95.7 ± 6.2
189.9	9	101.4 ± 3.0
Pooled data	64	100.0 ± 7.5

[a] Recovery was calculated as

$$\text{recovery (\%)} = \frac{(X_1 - X_0) \times 100}{A}$$

where X_0 and X_1 are measurements before and after addition of allantoin, and A is the amount of allantoin added.

Accuracy of the method

Accuracy of the method was assessed by examining the recovery of known quantities (31–158 μmol/l) of allantoin added to urine or plasma. Results are shown in Table I. The averaged (±S.D.) recovery was 95 ± 3.7% (n = 45) and 100 ± 7.5% n = 64) for urine and plasma samples, respectively. The recovery can also be calculated by regression approach, *i.e.* by comparing the responses (slope of regression line) of allantoin-A peak area (y) to the quantity of allantoin

(x, μmol/l) added to water (standard), urine or plasma. Results are: standard: $y = 5535\ x - 14361$ ($r = 0.999$, $p < 0.001$); urine: $y = 5406\ x + 225170$ ($r = 0.998$, $p < 0.01$); plasma: $y = 5274\ x + 309060$ ($r = 0.999$, $p < 0.001$). Recovery for allantoin added to these urine and plasma samples can therefore be calculated as 98 and 95%, respectively. The incomplete recovery implies that in urine or plasma (but not in standard), a fraction of a certain allantoin-deriving substance did not proceed to the final formation of GLX-DNPH. However, it is important to note that the recovery factors for both urine and plasma were close to 1 and constant. With urine samples, correction for recovery factor (95%) could be built into the calculation.

The lowest concentration of allantoin that gave a reproducible integration was 5 μmol/l although samples were usually diluted to have a concentration of between 30 and 320 μmol/l. Variability of repeated analysis was small. When six samples were each analyzed (derivatization and injection) seven times on different days, the assay-to-assay coefficient of variation (C.V.) derived from the seven measurements of allantoin-A peak area averaged 2.8 ± 0.80%. The within-assay C.V. based on three samples repeated five times (derivatization and injection) was 0.6 ± 0.06%. The C.V. for repeated injections was 0.2 ± 0.06% based on three samples each with five injections.

Like the method of Young and Conway [2], this method effectively measures allantoin as GLX. Therefore, the presence of original GLX in the sample would give an overestimation of allantoin content. The content of original GLX was measured in 24 urine samples and 14 plasma samples. All samples were carried through the described derivatization procedure except that water was used instead of NaOH. With all urine samples, the amount of GLX originally present was negligible since the allantoin-A and -B were undetectable (see Fig.1b). With the plasma samples, the allantoin-A and -B peaks were small (see Fig. 1c), equivalent to 8 ± 1.9 μmol/l allantoin ($n = 14$). Allantoin concentration in plasma of sheep and cattle is normally in the range 50–150 μmol/l [14].

As illustrated in Fig. 1c, plasma samples contained two other unidentified DNPH complexes (peaks C and D) that were of comparable concentrations with GLX-DNPH. These are presumably hydrazones of other keto acids [11]. The production of the precursors also involves hydrolysis under alkaline condition. Like GLX-DNPH, these complexes would also produce a red colour (absorbance at 520 nm) when made strong alkaline in the colorimetric assay of Borchers [10], thus making this assay non-specific for GLX-DNPH. Since the unidentified compounds were well separated from GLX-DNPH using the current chromatographic conditions, their presence should not affect the peak determination of GLX-DNPH. It is useful to note that the methods of Young and Conway [2] and of Borchers [10] are both liable to strong interference by formaldehyde (which may be present in urine of ruminants receiving formaldehyde-treated feedstuffs) and the current method is void of this problem.

The advantages of this method are as follows. (1) Through the pre-column derivatization, allantoin is converted to its derivatives that can be separated and measured easily. The chromatographic separation is complete and rapid (96 samples per day) and uses a widely available C_{18} column. (2) The derivatization procedure used is both simple and robust (stable reagents and reaction products). This method is accurate with good reproducibility and provides an alternative to other methods of allantoin determination.

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