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Short Communication

Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography

J. Balcells*, J. A. Guada and J. M. Peiró

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Miguel Servet, 177, Zaragoza 50013 (Spain)

D. S. Parker

Department of Biological and Nutritional Sciences, Faculty of Agriculture and Biological Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU (UK)

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ABSTRACT

A high-performance liquid chromatographic method is described for the separation and quantification of allantoin and oxypurines in plasma and urine samples. Urine was analyzed directly and plasma after acid deproteinisation with perchloric acid. Separation and quantification of purine derivatives was achieved using two Spherisorb ODS-5 column (250 mm × 4.6 mm I.D.) connected in series together with a $NH_4H_2PO_4$–$NH_4H_2PO_4$–acetonitrile (80:20) gradient and monitoring the effluent at 205 nm. The average recoveries of standard compounds added to urine and plasma samples were 96 and 97%, respectively, using allopurinol as internal standard. The within-day variability was less than 7% and the day-to-day coefficient of variation less than 11% indicating a good precision of the method.

INTRODUCTION

In ruminants dietary nucleic acids are extensively degraded in the rumen and duodenal purines appear to originate predominantly from rumen microorganisms [1]. This has led to a renewed interest in the determination of urinary purine excretion as a possible indicator of the amount of purine and, hence, microbial protein flowing to the small intestine [2]. The metabolism of purine bases to allantoin consists of a series of reactions involving hypoxanthine, xanthine, uric acid and allantoin, all of which are excreted in urine, with allantoin constituting the greatest proportion.

In previous studies allantoin has been determined in urine by a colorimetric method [3] based on the quantification of the aldehyde group of glycoxylic acid formed following hydrolysis of allantoin. Xanthine and hypoxanthine are determined in the same way after enzymatic conversion to uric acid [4] or allantoin [5], respectively.

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These methods, however, lack specificity due to possible overestimation of the presence of interfering compounds [3] and are very sensitive to the analytical conditions employed.

Several high-performance liquid chromatographic (HPLC) methods have been developed for the quantification of nucleotide, nucleoside and purine bases [6–10] but there have been no reports which include purine derivatives and allantoin. This paper describes a method for the quantitative determination of oxypurines and allantoin in biological fluids.

EXPERIMENTAL

Instrumentation

A Gilson dual-pump HPLC system (Gilson Medical Electronics, Middleton, WI, USA) with automatic injector (Model 231 sample injector) was used for the analyses. Separations were achieved by using two Spherisorb ODS-5 reversed-phase columns (250 mm × 4.6 mm I.D., Phase Separations, Queensferry, UK) connected in series.

Materials

All purine standards were purchased from Sigma (Poole, UK). Urine was collected daily from sheep in metabolic cages into sulphuric acid (10%) to maintain a pH of less than 3. Blood samples were obtained from catheters chronically implanted in the carotid artery.

Buffer preparation

An initial stock solution of 0.1 *M* $NH_4H_2PO_4$ was prepared and kept at 4°C. Working buffers were prepared daily.

Buffer A. Buffer A was prepared by diluting 100 ml of stock solution to 1 l with bidistilled water, the pH adjusted to 4.0 with phosphoric acid and filtered through a 0.45-μm filter (Millipore, Bedford, MA, USA).

Buffer B. Buffer B was prepared by diluting 100 ml of stock solution to 800 ml with bidistilled water, the pH adjusted to 4.0 and adding 200 ml of acetonitrile. Before use the buffer was stirred for 3 h at room temperature and filtered as described above.

Chromatographic conditions

System I. For a complete analysis of allantoin, uric acid, xanthine and hypoxanthine in urine samples (20 μl), a 30-min linear gradient from 0 to 100% buffer B was run at 0.8 ml/min. After 40 min, the column was re-equilibrated for 20 min in 100% buffer A. The effluent was monitored at 205 nm with full-scale deflection set at 0.2 absorbance units.

System II. For a complete analysis of purine metabolites in plasma samples buffer B gradient followed the sequential steps 0% for 5 min, 0 to 2% in 2 min, 2 to 100% in 23 min, followed by the re-equilibration process described above. For plasma analysis the buffer pH was adjusted to 6.0.

Standard solution

Xanthine and uric acid were dissolved in alkalinized (pH > 10) hot water and allantoin, hypoxanthine and allopurinol in distilled water to a concentration of 0.5 m*M*. After neutralizing the xanthine and uric acid solution to pH 6 with phosphoric acid a mixture of equal volumes of each standard solution was used.

Sample preparation

Urine. Urine samples were diluted 1:20 with the stock buffer solution, filtered through a Millipore 0.45-μm filter (Millipore) and analyzed directly.

Blood. Blood samples were collected in heparinized tubes and immediately centrifuged for 5 min at 10 000 *g* (Microcentaur MSE). Plasma samples (1 ml) were pipetted into a microcentrifuge tube (Model 3810, Eppendorf, Hamburg, Germany) and cooled in an ice bath, 100 μl of perchloric acid (4 *M*) solution were added and the mixture was vortex-mixed for 1 min. After 10 min on ice, the suspension was centrifuged (5 min at 10 000 g), the supernatant pipetted into another tube and the pH adjusted (using Bromothymol Blue as pH indicator) to 6–7 with an ice cold solution of 4 *M* potassium hydroxide. After 10 min the precipitated potassium perchlorate was removed by centrifugation (10 min at 2000 *g*) and the extract was analyzed in the same day. Allopu-

rinol was used as internal standard and added to each sample before the deproteinization (100 μl, 1.1 m*M*).

RESULTS AND DISCUSSION

Figs. 1 and 2 show the separation of allantoin and oxypurines in urine and plasma samples using systems I and II, respectively.

The major analytical problem in the present work was obtaining a suitable separation of allantoin. Urine and plasma samples contain several components with a similar high polarity and UV absorption. Satisfactory allantoin separation in urine samples was obtained using a long column, to increase the theoretical plates, together with a low molarity and pH of the eluent in order to increase retention time of the interfering compounds [11]. However, in plasma samples, the optimal peak resolution between allantoin and interfering compounds was obtained when buffers were adjusted to pH 6.0. The pH also had a significant influence on the retention times of uric acid (Fig. 2) in agreement with the results of Putterman *et al.* [12]. The inclusion of an acetonitrile gradient resulted in convenient retention times for the several compounds studied and avoided accumulation of non-polar compounds on the column, so that after analysis of 100 urine samples the quality and retention times of the peaks was still excellent.

In previous studies nucleic acid components (nucleotide, nucleoside and purine bases) have usually been monitored at 254 nm. However, these compounds also possess a strong chromophore in the region 206–210 nm which is coincident with the maximum allantoin UV absorbance (195 nm); this enabled determination of allantoin and allantoin precursors at the same wavelength (205 nm).

In complex extraction procedures with, for example, blood samples, it is essential to use an internal standard to correct for recovery and analytical variability. Various compounds which were tested in the present study could have been used as internal standards, for example 6-mer-

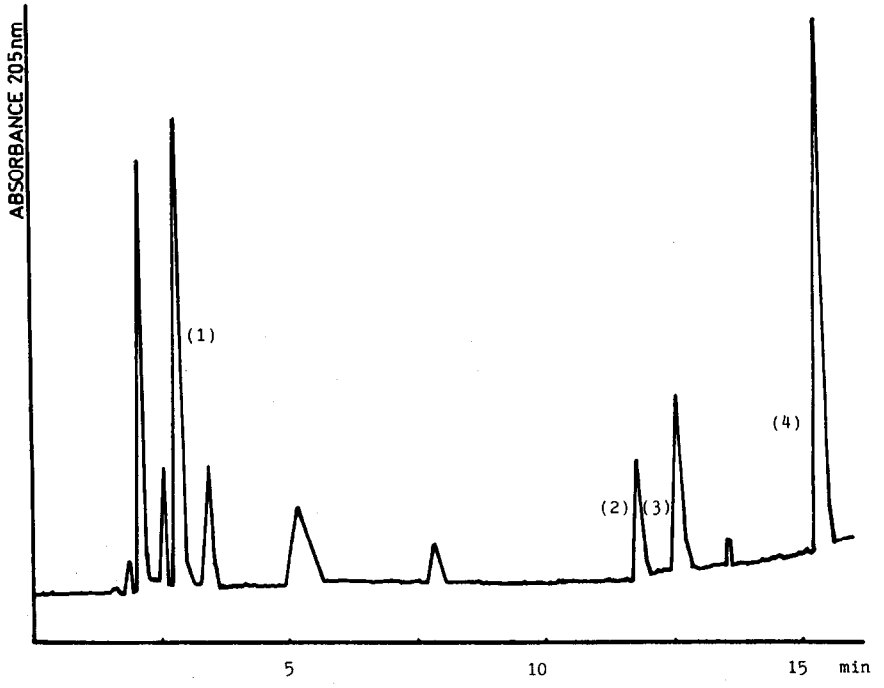

Fig. 1. Chromatogram of sheep urine sample obtained using system I. Peaks: 1 = allantoin; 2 = uric acid; 3 = hypoxanthine; 4 = internal standard.

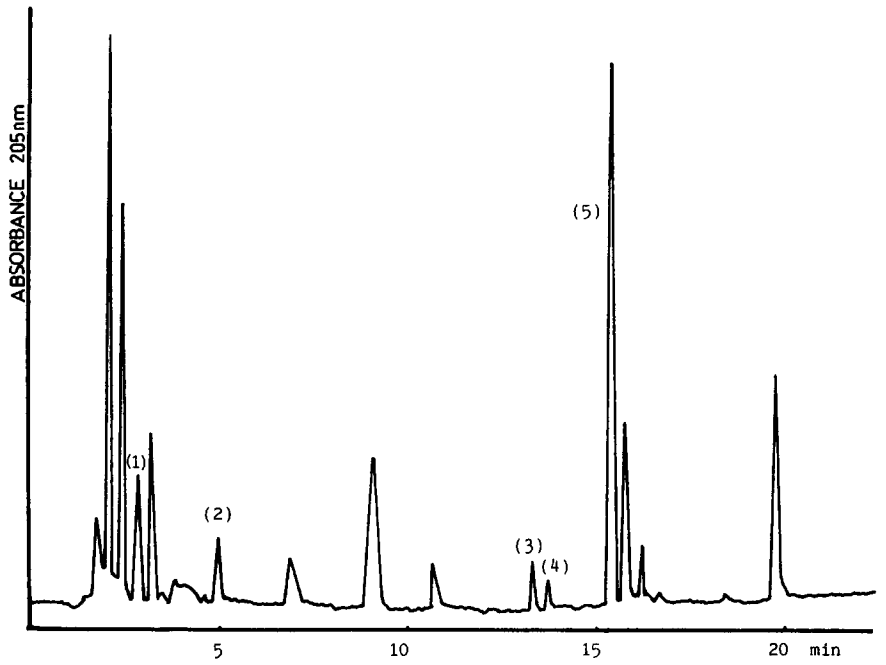

Fig. 2. Chromatogram of sheep plasma sample obtained using system II. Peaks: 1 = allantoin; 2 = uric acid; 3 = hypoxantine; 4 = xanthine; 5 = internal standard.

captopurine, theophylline, oxipurinol, orotic acid and allopurinol. Some of them, however, showed specific disadvantages: theophylline and 6-mercaptopurine are highly toxic and exhibit excessive retention times; orotic acid was detected in steer blood samples and oxipurinol was poorly resolved on the column. Allopurinol showed good chemical stability and peak resolution in both urine and blood samples, moreover, the spectrum for allopurinol possesses two maxima at 205 and 251 nm and was therefore suitable for use at the wavelength for nucleic acid derivative detection. The drug allopurinol can be transformed to oxipurinol by xanthine oxidase (EC 1.2.3.2) and this enzyme has been reported in bovine plasma [13]. However, by maintaining blood samples continuously in an ice bath, before deproteinization, this enzymatic transformation is negligible. In our results, neither significant losses of allopurinol, nor oxopurinol formation as result of the enzymatic action of xanthine oxidase, were observed.

The relationship between the concentration and peak area was linear over a wide range of concentrations (10–150 μM) and from the data were calculated the following equations and correlation coefficients: $y = -0.45 + 1.6x$ ($r^2 = 0.998$) for allantoin; $y = -0.41 + 5.1x$ ($r^2 = 0.997$) for uric acid; $y = 0.65 + 6.5x$ ($r^2 = 0.998$) for hypoxanthine; and $y = 2.04 + 6.6x$ ($r^2 = 0.997$) for xanthine where y is the peak area and x purine concentration.

Standard recovery of purine derivatives added to urine and plasma samples based on the ratio purine base peak area/internal standard area are presented in Table I. The day-to-day precision of the method was obtained by processing five aliquots of urine and plasma samples spiked with various concentration of the standard (range 25–150 μmol) on five consecutive days. Within-assay coefficients of variations were calculated by repeatedly processing ($n = 10$) aliquots of spiked urine and plasma samples. There was no difference between total recovery estimated from the relationship between concentrations and peak area or by using internal standard for urine sam-

TABLE I

RECOVERIES OF STANDARD PURINE METABOLITES ADDED TO URINE AND PLASMA SAMPLES CALCULATED ON THE RATIO OF THE SAMPLE PEAK/INTERNAL STANDARD (ALLOPURINOL) AREAS AND PRECISION OF THE METHOD OBTAINED IN WITHIN-DAY (n = 10) AND DAY-TO-DAY (n = 5) MEASUREMENTS

Compound	Within-day				Day-to-day			
	Mean recovery (%)		Coefficient of variation (%)		Mean recovery (%)		Coefficient of variation (%)	
	Urine	Plasma	Urine	Plasma	Urine	Plasma	Urine	Plasma
Allantoin	96.7	98.6	3.21	4.61	96.3	96.7	4.85	5.14
Uric acid	94.6	96.5	6.64	7.04	92.5	94.3	7.34	8.13
Hypoxanthine	97.3	98.6	2.42	3.82	96.7	96.6	3.22	3.64
Xanthine	97.4	98.5	3.26	5.60	98.3	95.5	6.43	10.18

ples. In plasma samples, however, absolute recoveries based upon peak area significantly underestimated the recovery when compared to values based upon internal standard calculations (absolute recoveries for allantoin, uric acid, hypoxanthine and xanthine in plasma samples were 89.3, 87.3, 82.7 and 86.6%, respectively, as a mean in within- and between-day measurements).

In the system developed in this study the detection limit at a signal-to-noise ratio of 3 in urine and plasma samples was 0.06 nmol for allantoin, xanthine and hypoxanthine and 0.1 nmol for uric acid.

The HPLC method described in this study enabled rapid analysis of purine derivatives in biological samples without the disadvantages inherent in colorimetric analyses. Application of the method in the study of purine metabolism in ruminants should provide further evidence of the influence of nutritional manipulation on microbial protein supply to the small intestine.

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