Simultaneous determination of purine derivatives in urine by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatography method for the analysis of allantoin, uric acid, hypoxanthine and xanthine in urine of sheep is described. Urine samples were analyzed directly after dilution. The combination of HPLC reversed phase C_{18} columns with monitoring the effluent at 205 nm provides a relatively rapid and simple analytical tool for studying purine derivatives in the urine of ruminants. Separation and quantification of purine derivatives was achieved using three C_{18} columns connected in a series together with a $NH_4H_2PO_4 - NH_4H_2PO_4$ and methanol (95:5) gradient. The average recoveries of standard compounds added to urine samples were satisfaction (96-103%). The low value of the intra-assay coefficient of variation and high recovery point to the satisfactory precision and accuracy of the reported method.

KEY WORDS: allantoin, uric acid, hypoxanthine, xanthine, urine, determination, HPLC

INTRODUCTION

The final products of nucleic acid purine base catabolism in most mammals are allantoin, uric acid, hypoxanthine and xanthine (Chen et al., 1990 a,b, 1993, 1996; Balcells et al., 1993; Susmel et al., 1994). These purine derivatives (PD) are

excreted in urine, in which allantoin constitutes the greatest proportion (about 85%) of total PD (Chen et al., 1993). The purine derivatives originate mainly from the nucleic acids of rumen microorganisms, so measurements of urinary PD levels provide an indication of the amount of microbial protein supplied to ruminants and is well correlated with organic matter or digestible organic matter intake (Antoniewicz et at., 1985; Lindberg et al., 1991; Balcells et al., 1993; Chen et al., 1993).

Although some authors have proposed allantoin excretion as an index of the microbial protein supply to the small intestine in ruminants, a more appropriate indicator might be the excretion of other or total purine derivatives (Topps et al., 1965; Zinn et al., 1986), particularly since ruminant urine also contains considerable amounts of uric acid, hypoxanthine and xanthine (Chen et al., 1990; Puchala et al., 1992). The main advantage of such an index is that it does not require the use of an invasive method to estimate microbial biomass supply to the duodenum. Several methods have been developed for measuring PD (Fujihara et al., 1987; Chen et al.,1990b, 1996; Balcells et al., 1993; Puchala et al., 1993; Czauderna and Kowalczyk, 1995), but there are few reports that include simultaneous determination of allantoin, uric acid, hypoxanthine and xanthine (Balcells et al., 1992).

The aim of the present paper was to present a simple procedure for PD determination in urine without derivatization, using HPLC-UV detection. The application of such a method in the study of purine metabolism in ruminants might provide further evidence of the influence of nutritional manipulation on microbial protein supply to the small intestine.

MATERIAL AND METHODS

Reagents

Methanol was purchased from POCH (Gliwice, Poland), while allantoin, uric acid, hypoxanthine and xanthine were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and purchased from POCH (Gliwice, Poland). Water was distilled and then deionized prior to use. HPLC-grade water was prepared using a Milli-Q system (Millipore, Toronto, Canada). The mobile phase (solvents A and B) was filtered through a 0.2 μm membrane filter (Millipore). The solvents were degassed by 10 min ultra-sonication prior to use.

HPLC configuration

A Waters 625 LC system (including a controller and two pumps) was used. The apparatus consisted of a Waters 712 WISP autosampler, turnable Waters Model 486 absorbance detector and computer data handling system (all equipment from Waters, Millipore, MA, USA).

The analytical procedure, collection and data integration methods were developed using Millennium 2001 software and a Pentium 5P60 computer. Separations were achieved by using two Nova-Pak C_{18} – columns (4 μm, 150 mm x 3.9 mm I.D., Waters, Millipore) and a Chrompack column – C_{18} (4 μm, 100 mm x 3 mm I.D., Chrompack, The Netherlands) connected in a series. A Waters cartridge (10 mm x 6 mm I.D., Millipore, MA, USA) containing reversed phase C_{18} (30-40 μm) pellicular packing material was used as the precolumn.

Analytical solvents and gradient composition

A binary gradient program (Waters curvilinear program) was used for the complete analysis of allantoin, uric acid, hypoxanthine and xanthine in urine samples. The following eluents were used: (A) – $NH_4H_2PO_4$ (0.0025 M) buffered to pH 3.5 with 10% phosphoric acid; (B) – the solvent A and methanol (95/5 v/v). For the analysis of urinary purine metabolites, the solvent B gradient was followed by the sequential steps: 0% the solvent B at 0 min, 80% at 21 min (linearly increased from 20 min, line No. 6), 0% at 30 min (linearly decreased from 29 min, line No. 6). The total flow-rate was 0.4 ml/min (the system pressure was 2200 ± 50 psi). After 30.1 min, the columns were re-equilibrated for 45 min in 100% solvent A at a flow-rate of 0.5 ml/min (the maximum system pressure was 2870 ± 50 psi). Injection volumes were 5 or 10 μl. The detector was operating at 205 nm with an attenuation of 0.050 a.u.f.s (the absorbance unit full scale). The separation was performed at 17-27^{0}C.

Standards

Appropriate amounts of purine derivatives were dissolved in the solvent A. To provide calibration for urine samples, sets of purine derivative standards were used.

Urine was collected from sheep in metabolic cages and as a routine method of preservation, urine samples were acidified to a pH below 3 with 1 M H_2SO_4. For analysis urine samples were mixed and then diluted 1:40 or 1:20 with solvent A. All standards and urine samples were filtered through a 0.2 μm filter (Cole Parmers) into an autosampler vial and analyzed directly.
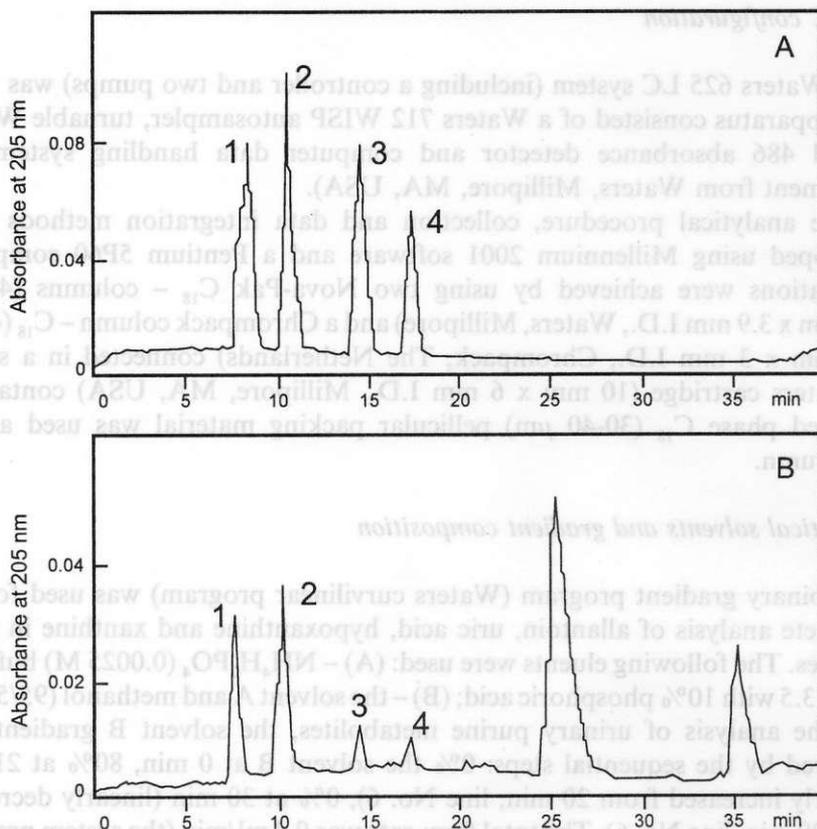
Figure 1. Chromatograms for standard (A) and urine (B). The peaks and their retention times are: (1) – allantoin at 8.2 min, (2) – uric acid at 10.7 min, (3) – hypoxanthine at 14.9 min and xanthine at 17.4 min

RESULTS AND DISCUSSION

Good separation of all purine derivatives-allantoin, uric acid, hypoxanthine and xanthine in urine samples was achieved during the chromatographic run owing to the use the gradient program and monitoring the effluent at 205 nm. Urine contains several components with a similar high polarity and UV absorption. Thus, a long column together with a low molarity and pH of the eluent were needed to achieve satisfactory separation of PD in urine samples, and to increase the theoretical plates so as to increase the retention time of the interfering species. In our procedure, the allantoin peak was eluted at 8.2 ± 0.6 min, uric acid at 10.7 ± 0.8 min, hypoxanthine at 14.9 ± 0.9 min and xanthine at

Summarized results of recoveries (R %, i.e. mean ± SD)[1] of standard purine metabolites added to sheep urine and concentrations (C) of added purine derivatives, μM

	Allantoin		Uric acid		Hypoxanthine		Xanthine	
C	187.2	(2)[2]	136.7	(2)	110.2	(4)	75.0	(3)
R	102.2 ± 3.8		104.1 ± 4.6		98.2 ± 1.4		102.2 ± 2.8	
C	124.8	(4)	91.2	(3)	73.5	(7)	50.0	(4)
R	101.3 ± 3.4		97.7 ± 3.8		99.4 ± 1.1		101.9 ± 1.9	
C	93.6	(2)	68.4	(2)	55.1	(5)	37.5	(4)
R	98.7 ± 4.1		99.2 ± 3.3		100.5 ± 0.9		95.1 ± 4.6	
C	62.4	(3)	45.6	(3)	36.7	(7)	25.0	(4)
R	96.8 ± 4.7		103.8 ± 4.3		100.7 ± 1.8		102.6 ± 3.5	
C	46.8	(1)	34.2	(1)	27.6	(2)	18.7	(2)
R	96.2		93.8		103.4 ± 6.5		96.1 ± 5.4	
C	12.5	(2)	9.1	(2)	7.3	(3)	5.0	(3)
R	98.3 ± 5.3		95.3 ± 4.4		103.8 ± 3.7		97.1 ± 6.1	
Pooled data	98.7 ± 4.7		98.8 ± 4.1		101.1 ± 2.8		98.7 ± 4.2	

[1] recovery was calculated as:

$$R\,(\%) = \frac{(S_1 - S_0) \times 100\%}{S}$$

where S_0 and S_1 are measurement before and after addition of standard purine derivatives, and S is the amount of added purine derivatives

[2] number of replicates

17.4 ± 0.9 min (Figure 1). Good linearity was obtained for a wide range of PD standards concentrations and the following equations and correlation coefficients were calculated from the data:

– for allantoin (the range of concentration: 37.4 – 1496 μM):
    $y = 2.992 \cdot 10^{-7} \cdot S_n + 0.002,$ $R = 0.9999$

– for uric acid (the range of concentration: 27.3 – 1092 μM):
    $y = 8.274 \cdot 10^{-8} \cdot S_n - 0.010,$ $R = 0.9989$

– for hypoxanthine (the range of concentration: 22.0 – 880 μM):
    $y = 5.565 \cdot 10^{-8} \cdot Sn + 0.005,$ $R = 0.9999$

– for xanthine (the range of concentration: 14.9 – 596 μM):
    $y = 1.104 \cdot 10^{-7} \cdot S_n + 0.001,$ $R = 0.9993$

where: S_n and y are the peak area and the purine concentration (μM), respectively.

The within-assay coefficients of variations (C.V.) derived from the measurements of purine derivatives in urine samples

Allantoin		Uric acid	Hypoxanthine	Xanthine
CV^1 % 4.7	⟨•⟩	4.2	2.8	3.4
CV^2 % 3.8		3.4	2.6	2.9

[1] the within-assay C.V. based on three samples repeated three or two times (processing and injection)
[2] the within-assay C.V. based on three samples each with three injection

The accuracy of the method was assessed by examining the recovery of known quantities of allantoin, uric acid, hypoxanthine and xanthine added to urine samples. Recoveries (R) of standard purine metabolites added to the urine and the day-to-day precision of the method are presented in Table 1. It can be seen that PD added to urine were recovered satisfactorily (96-103%). The intra-assay coefficients of variation (CV) were calculated by repeatedly processing aliquots of spiked urine sample (Table 2) while the lowest concentrations of purine derivatives that gave a reproducible integration were: 9 μM for allantoin, 2 μM for uric acid, 1.5 μM for hypoxanthine and 3.8 μM for xanthine.

It can be concluded that the presented HPLC method for assaying purine derivatives makes it possible to separate and quantify allantoin, uric acid, hypoxanthine and xanthine in urine samples. The urine samples can be analyzed directly after dilution. Moreover, the method described in this study enables simple and simultaneous determination of all PD without the disadvantages inherent in pre- or post-column derivatization procedures. The purine metabolites are detected at short wavelengths, as they possess a strong chromophore in the region 206-210 nm (Balcells et al., 1992; Chen et al., 1996). All purine derivatives were completely resolved in about 19 min, however, the total run time, including column re-equilibration, was 75 min.

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STRESZCZENIE

Jednoczesne oznaczanie pochodnych purynowych w moczu metodą HPLC

Opisano metodę oznaczania allantoiny, kwasu moczowego, hipoksantyny i ksantyny w moczu owiec przy użyciu zestawu HPLC. Mocz analizowano bezpośrednio po doprowadzeniu do pH 3 oraz odpowiednim rozcieńczeniu. Pochodne purynowe rozdzielono wykorzystując trzy kolumny z odwróconą fazą (C_{18}) poprzez elucję gradientową i monitorowanie przy 205 nm. Zmiany w składzie fazy ruchomej (0,4 ml/min) realizowano programowo przez zmiany względnej prędkości podawania dwóch roztworów – A (0,025 M $NH_4H_2PO_4$) i B – (roztwór A i metanol: 95/5 v/v). Odzysk pochodnych purynowych dodanych do moczu był niemal zupełny. Mała wartość współczynnika zmienności w obrębie próby (przygotowanie i iniekcja) oraz iniekcji tej samej próby, wysoki odzysk oznaczanych związków (96-103%) dowodzi, że prezentowana prosta metoda jest dostatecznie dokładna.