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Determination of creatinine and purine derivatives in ruminants' urine by reversed-phase high-performance liquid chromatography

J. A. Resines

Departamento de Física, Química y Expresión Gráfica, Universidad de León, 24071-León (Spain)

M. J. Arín and M. T. Díez*

Departamento de Bioquímica y Biología Molecular, Universidad de León, 24071-León (Spain)

ABSTRACT

A procedure is described for the rapid and simultaneous determination of allantoin, creatinine, uric acid, hypoxanthine and xanthine in sheep urine. Separation was achieved on a Novapak C_{18} column under isocratic conditions. The mobile phase was potassium phosphate buffer (10 m*M*, pH 4.0). A flow-rate of 0.5 ml/min, detection at 218 nm and a column temperature of 25°C were employed with a total analysis time of less than 15 min. Detection limits for allantoin, creatinine, uric acid, hypoxanthine and xanthine were 1.0, 0.5, 0.5, 0.5 and 0.2 μg/ml, respectively, at a signal-to-noise ratio of 3 in a 20-μl injection volume of tenfold-diluted urine. This sensitivity permits the precise determination of these compounds in ruminants' urine.

INTRODUCTION

In ruminants, purines are metabolized in a series of reactions to form allantoin, uric acid, hypoxanthine and xanthine. These compounds, present in urinary excretion, were proposed as an index of their nutritive status in each phase of the productive cycle [1].

Allantoin is the main excretion product from purine metabolism and it has been shown [2] that urinary excretion in sheep was significantly correlated with the nucleic acid concentration in rumen fluid [3,4].

Urinary creatinine excretion was correlated with the live mass within a wide range of body mass [5]. Creatinine could be useful as an internal marker to make quantitative predictions of metabolic processes in intact animals [4].

Various methods have been described for the determination of allantoin and creatinine in biological fluids. Most procedures are based on colorimetric reactions. The traditional colorimetric analysis of allantoin in urine is based on the Rimini–Schryver reaction described by Young and Conway [6]. Lindberg and Jansson [7] and Chen *et al.* [8] described a method that adapted this reaction to the Technicon AutoAnalyzer.

For creatinine determination, the method most widely employed in clinical laboratories is the Jaffe alkaline picrate procedure [9,10]. This method requires several steps and it has been reported that it can give overestimated values of creatinine owing to interferences by endogenous and exogenous pseudo-creatinine chromogens [11].

Uric acid, hypoxanthine and xanthine have long been measured colorimetrically. More specific enzymatic techniques have been widely applied; both methods have problems [12,13] with intereferences from other compounds presents in biological fluids.

Reversed-phase high-performance liquid chromatographic (RP-HPLC) assays of allantoin in biological fluids proved to be good alternatives

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[14,15]. In recent years, several chromatographic methods for the determination of creatinine have been described. Approaches include cation-exchange column chromatography [16], ion-pair chromatography [17] and reversed-phase chromatography [18,19]. A series of papers reported HPLC methods for the determination of uric acid, hypoxanthine and xanthine. Particularly anion-exchange liquid column chromatography for routine clinical laboratory use appears far too time consuming [20].

Currently reversed-phase techniques have been most widely employed. Some workers have reported gradient elution techniques [21,22] while others recommended isocratic elution [23].

For the study of ruminant metabolism, a simple and rapid method is needed for measuring urinary allantoin, creatinine, uric acid, hypoxanthine and xanthine simultaneously. In this paper, an RP-HPLC method for this purpose is described.

EXPERIMENTAL

Reagents

Allantoin was obtained from Sigma (St. Louis, MO, USA). Creatinine, uric acid, hypoxanthine and xanthine were purchased from Merck (Darmstadt, Germany) and used without further purification. Other chemicals were of the highest purity commercially available. Methanol was of HPLC grade, obtained from Carlo Erba (Milan, Italy). Water was previously distilled and purified with a Milli-RO 15 reagent-grade water system (Millipore, Bedford, MA, USA).

Urine samples

Urine samples were centrifuged and filtered through a Millex-HV 0.45-μm pore size filter (Millipore) and diluted tenfold (or more when the concentrations in samples were high with distilled water. A 20-μl volume of the filtrate was injected into the HPLC column. Urine samles were stable for several weeks when stored at -20°C.

Standard solutions

Stock solutions of all compounds (1 mg/ml) were prepared by dissolving pure standards in water and were stored at 4°C for 1 month. Working standard solutions, were prepared weekly, 40 μg/ml for allantoin, 60 μg/ml for creatinine and 20 μg/ml for uric acid, hypoxanthine and xanthine, by diluting the stock solutions with water; a 20-μl aliquot of these solutions was used daily as a control to check the retention time and all other conditions of the HPLC procedure. A series of working standards were prepared by dilution of each of the stock solutions with water.

Calibration graphs were prepared over the concentration range 20–400 μg/ml for allantoin, 10–200 μg/ml for creatinine and 5–35 μg/ml for uric acid, hypoxanthine and xanthine.

Quantification was achieved by regression analysis of the peak areas of each compounds against concentration. Triplicate injections of each concentration were made.

Instruments

HPLC analyses were performed with a Waters (Milford, MA, USA) Model 600E system equipped with a Waters Model 484 UV detector. Quantification was based on integration of peak areas using a Waters Model 745B integrator.

Chromatographic conditions

A Novapak C_{18} reversed-phase column (30 cm $\times$ 3.9 mm I.D., 4-μm particles) (Waters) was used. The mobile phase was 10 m*M* potassium phosphate buffer (pH 4.0). Before use, the mobile phase was filtered through an HA 0.45-μm pore size filter (Millipore) and further degassed by sonication. The flow-rate was 0.5 ml/min, the column was maintained at 25°C and the absorbance detector was set at 218 nm.

Compound peaks were identified by their retention times and co-elution with authentic standards and quantified by comparison of the peak areas of the samples with those of authentic standards.

The purity of the compound peaks was tested by comparison of the peak areas obtained at wavelengths of 218 and 230 nm.

RESULTS AND DISCUSSION

Creatinine and allantoin have similar polarity and it is very difficult to separate them in biological fluids. In previous work [15] we found the optimum chromatographic conditions for the separation of the creatinine peak from the allantoin peak [mobile phase, 10 m*M* potassium phosphate buffer, (pH

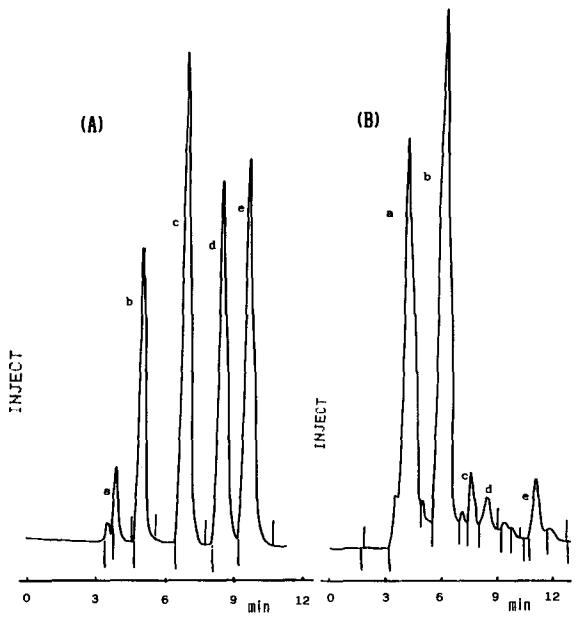

Fig. 1. (A) Chromatographic separation of standard solutions. (B) Chromatogram of tenfold-diluted sheep urine. Peaks: a = allantoin; b = creatinine; c = uric acid; d = hypoxanthine; e = xanthine.

4.0); flow-rate, 0.5 ml/min; temperature, 25°C; wavelength, 218 nm]. This method was applied successfully to the determination of uric acid, hypoxanthine and xanthine. Previously we have verified that there is little influence of the buffer concentration and column temperature on their separation. For this reason these conditions were applied to the simultaneous determination of the five metabolites with good resolution.

Fig. 1 shows chromatograms obtained for standard solutions and urine samples. The retention times for allantoin, creatinine, uric acid, hypoxanthine and xanthine were *ca.* 3.9, 5.7, 7.2, 8.3 and 10.8 min, respectively. At a signal-to-noise ratio of 3, the detection limits for tenfold diluted urine were 1.0, 0.5, 0.5, 0.5 and 0.2 μg/ml, respectively with a 20-μl injection.

In all instances, a linear relationship between the peak area and the concentration in urine was obtained for the ranges of concentrations tested, *i.e.*, 20–400 μg/ml for allantoin, 10–200 μg/ml for creatinine and 5–35 μg/ml for uric acid, hypoxanthine and xanthine. The equations calculated were $y = 0.029\,x + 0.263$ for allantoin [15], $y = 0.167\,x + 0.844$ for creatinine [15], $y = 0.058\,x - 0.009$ for uric acid, $y = 0.117\,x - 0.135$ for hypoxanthine and $y = 0.119\,x - 0.072$ for xanthine (y = peak area $\cdot 10^{-6}$; x = concentration). In all instances the correlation coefficients were greater than 0.99.

The standard addition method was used to check for chemical interferences. The equations calculated with the standard addition method applied to urine were $y = 0.026\,x + 14.091$ for allantoin [15], $y = 0.162\,x + 3.992$ for creatinine [15], $y = 0.057\,x + 0.969$ for uric acid, $y = 0.129\,x + 0.811$ for hypox-

TABLE I

INTER-DAY PRECISION AND ACCURACY OF THE DETERMINATION OF URIC ACID, HYPOXANTHINE AND XANTHINE IN SHEEP URINE

Compound	Concentration added (μg/ml)	Concentration found (μg/ml) (mean ± S.D.; $n = 3$)	R.S.D. (%)	Relative error (%)
Uric acid	10	10.9 ± 0.2	1.7	9.4
	15	15.9 ± 0.2	1.2	5.8
	20	20.7 ± 0.1	0.5	3.6
Hypoxanthine	10	11.0 ± 0.2	1.6	9.6
	15	15.7 ± 0.1	0.8	4.4
	20	21.1 ± 0.1	0.5	5.3
Xanthine	5	5.0 ± 0.1	2.1	1.2
	8	8.1 ± 0.1	1.2	1.0
	10	10.7 ± 0.1	0.9	6.9

anthine and $y = 0.126\,x + 0.203$ for xanthine. The slopes of the calibration and standard addition graphs were similar for each compound.

For each analyte, the inter-day precision and accuracy were determined by analysing three times per day for ten days diluted urine samples spiked at three concentrations. The results for uric acid, hypoxanthine and xanthine are given in Table I.

The results obtained for allantoin and creatinine were given in a previous paper [15]; for these two compounds, the relative standard deviation (R.S.D) varied between 1.2 and 2.2% for allantoin and between 1.1 and 2.5% for creatinine.

The recovery was determined by triplicate analyses of urine samples spiked with standards of the metabolites at concentrations ranging from 80 to 320 μg/ml for allantoin [15], from 20 to 120 μg/ml for creatinine [15], from 5 to 30 μg/ml for uric acid and hypoxanthine and from 2 to 15 μg/ml for xanthine. The recoveries were 97.9 ± 1.7% (R.S.D. = 1.8%) for allantoin [15], 99.1 ± 0.6% (R.S.D. = 0.6%) for creatinine [15], 93.6 ± 2.4% (R.S.D. = 2.5%) for uric acid, 106.5 ± 1.8% (R.S.D. = 1.7%) for hypoxanthine and 103.8 ± 1.2% (R.S.D. = 1.1%) for xanthine.

In conclusion, we have developed a sensitive RP-HPLC method for the simultaneous determination of allantoin, creatinine, uric acid, hypoxanthine and xanthine. The final chromatographic conditions adopted were a compromise between analysis time, peak shapes and symmetry and the resolution of these compounds from interefering substances. We consider that this method may be proposed as a possible reference method.

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