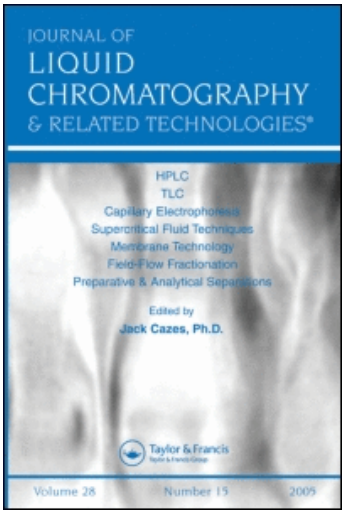

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 29 September 2003

To cite this Article Moral, P. García del , Díez, M. T. , Resines, J. A. , Bravo, I. G. and Arín, M. J.(2003) 'Simultaneous Measurements of Creatinine and Purine Derivatives in Ruminant's Urine Using Ion-Pair HPLC', Journal of Liquid Chromatography & Related Technologies, 26: 17, 2961 — 2968
To link to this Article: DOI: 10.1081/JLC-120025056
URL: http://dx.doi.org/10.1081/JLC-120025056

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 17, pp. 2961–2968, 2003

Simultaneous Measurements of Creatinine and Purine Derivatives in Ruminant's Urine Using Ion-Pair HPLC

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ABSTRACT

A new and sensitive ion-pair RP-HPLC method was developed for concomitant measurement of the creatinine and purine derivatives (PD): allantoin, uric acid, and hypoxanthine using allopurinol as internal standard. Urine was chromatographed on a Symmetry C_{18} column, using UV detection. Elution with a gradient of methanol in potassium phosphate buffer, pH = 4, containing 3 mM 1-octane-sulfonic acid, sodium salt yielded sharp, well-resolved peaks within 25 min. Application of this method permits the precise determination of these compounds in sheep urine.

Key Words: Creatinine; Purine derivatives.

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DOI: 10.1081/JLC-120025056
1082-6076 (Print); 1520-572X (Online)

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INTRODUCTION

Quantification of microbial protein supply is necessary to all ruminant protein evaluation systems. This information is essential for accurate protein feeding. Microbial protein supplies are generally evaluated using either internal or external microbial markers. Use of such an approach is unsatisfactory due to the requirement for fistulated animals. New approaches of rumen diagnostics based on the measurement of a compound that derives from rumen and, which appears in urine, have been developed in order to avoid the invasive methods.[1] A number of studies, have demonstrated that measurements of urinary excretion of allantoin and other purine derivatives (PD)[2,3] could potentially be used as the basis of a non-invasive index of microbial protein supply in sheep. Accurate assessment of urinary purine metabolite excretion in some ruminants requires a total urine collection. Creatinine could be useful as an internal marker of urinary output in sheep, allowing the collection of spot urine samples as an alternative to total collection.[4] The use of the index of the total PD and/or allantoin : creatinine can be an alternative to get the total collection of urine.[5] This proposition is based on the hypothesis that the creatinine excreted is constant and has a minimal variation that would allow its use as an internal marker to the estimation of the total volume of urine per day. Some authors[6,7] suggest that it would be enough to measure PD/allantoin, but it would be necessary that the excretion of allantoin were constant all day long, although some additional studies would be needed then.

The determination of these compounds has traditionally been done by spectrophotometric methods, although, chromatographics techniques have widely been used in its analysis lately.

Although most of the works published to determine these compounds use RP-HPLC with good results,[8–12] the difficulty of getting a good separation, which fits together time of analysis, accuracy, precision, and sensibility of the three most interesting compounds: creatinine, uric acid, and allantoin, has taken us to use ion-pair RP-HPLC, in order to improve, among all, the separation allantoin/creatinine, which due to its similar chemical structure is the most complex.

EXPERIMENTAL

HPLC Equipment

Waters Model (Milford, MA) 600-E instrument equipped with a Waters Model 717 plus injector and a 484 UV detector was used. The detection wavelength was set at 225 nm. The separation was carried out with a Symmetry C_{18}

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column (250 mm × 4.6 mm I.D., 5 μm) (Waters, Mildford, MA). Quantification was based on integration of peak areas using Borwin 1.5 software (JMBS Development).

Chemicals

The creatinine used for preparation of the standard solutions was obtained from Sigma (St. Louis, MO) and the other chemicals were purchased from Merck (Darmstadt, Germany) and used without further purification. Decane-, octane-, and hexanesulfonic acid (C10, C8, C6), sodium salts, were supplied by Sigma (St. Louis, MO). Methanol was of HPLC-grade obtained from Merck (Darmstadt, Germany). All other chemicals were the best grade commercially available. Deionized water was obtained with a Milli-Q apparatus (Millipore Bedford, MA).

Standard Solutions

Standard-stock solutions of creatinine, allantoin, hypoxanthine, and allopurinol (IS) (1000 mg/L) were prepared by dissolving them in water, and standard stock solution of uric acid (1000 mg/L) was prepared in 0.01 M sodium carbonate solution. The creatinine, allantoin, hypoxanthine, and allopurinol solutions were stable for at least one week, the uric acid solution for three days at 4°C. They were diluted with the elution solvent A described below to give standard solutions of various concentrations. For HPLC measurements standards of allantoin (50 mg/L); hypoxanthine (100 mg/L); uric acid (25 mg/L), and creatinine (100 mg/L), were prepared freshly each day.

The calibration range was 10–100 mg/L for allantoin, 5–50 mg/L for uric acid, 10–50 mg/L for hypoxanthine, and 10–200 mg/L for creatinine, obtained by dilution of standard-stock solutions with HPLC eluent A. In all samples the concentration of allopurinol (IS) was 100 mg/mL. The calibration graph was based on peak areas and was linear over the concentration range investigated. The peak area for every point was calculated as an average value of three injections, giving a relative standard deviation smaller than 3%.

Sample Preparation

Urine samples were centrifugated for 10 min at 3000*g* and filtered through a Millex-HV 0.45 μm pore size filter (Millipore, Bedford, MA) and diluted 10-fold with elution solvent A. Urine samples were stable for several weeks when stored at −20°C.

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Chromatographic Conditions

The mobile phase composition was phosphate buffer 10 mM with 3 mM 1-octanesulfonic acid, sodium salt, pH 4, mixed with methanol : eluent A (5%) and eluent B (20%). Before use, the mobile phase was always filtered through an HA 0.45 μm pore size filter (Millipore, Bedford, MA) and degassed by ultrasonication.

The gradient program was 0–13 min, 0–100% B, flow-rate of 0.5 mL/min; 13–25 min, 100–0% B, flow-rate of 1.5 mL/min; reequilibration time at 0% B, 10 min. The purity of every compound was tested by comparison of the peak areas obtained at wavelengths 225 and 254 nm.

The injection volume was 20 μL. The column temperature was set at 30°C.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

We tested different variables to optimize the simultaneous determination of creatinine and PD: alkyl chain length of the pairing-ion agent (C6, C8, C10), buffer concentration, pH and percentage of methanol of the mobile phase, and column temperature.

The retention time of creatinine, allantoin, and hypoxanthine decreased with the chain length of the pairing-ion molecules from C10 to C6, according to the previous results.[13] The retention time of uric acid does not show appreciable variations. The variation of creatinine and PD retention time was studied independently of the pH of the mobile phase in the range from 3.0 to 6.5. We adopted pH = 4, which gives a good separation and keeps the total analysis time short for these compounds. The effects of the buffer concentrations and column temperature were investigated, and we have verified that there is little influence of these parameters on the retention time of the analytes. According to these studies, we decided to adopt the chromatographic conditions specified under "Experimental," which represented a good compromise between good separation and reasonable analysis time.

The chromatograms resulting from the injection of pure standards and urine under the chromatographic conditions finally adopted, are presented in Fig. 1. The retention times for allantoin, uric acid, hypoxanthine, allopurinol (IS), and creatinine were 5.0, 6.7, 8.1, 11.5, and 19.2 min, respectively. A high reproducibility in the retention time was obtained with relative standard deviations (RSDs) less than 5%, in all cases studied.

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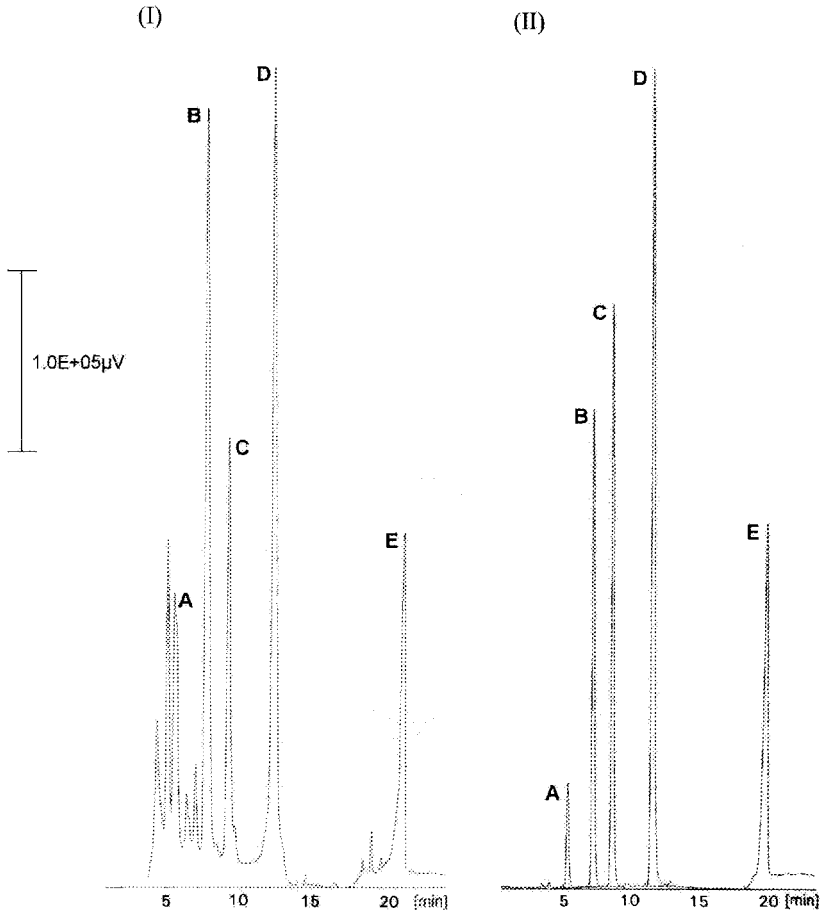

Figure 1. (I) Chromatogram of tenfold-diluted sheep urine. (II) Chromatographic separation of standard solutions. Peaks: A = Allantoin; B = Uric Acid; C = Hypoxantine; D = Allopurinol (IS); and E = Creatinine.

Analytical Variables

Linearity of the standards was checked by measuring various concentrations in the ranges 10–100 mg/L for allantoin; 5–50 mg/L for uric acid; 10–50 mg/L for hypoxanthine, and 10–200 mg/L for creatinine. Linear relationships between the peak areas and the concentrations tested were found. The equations calculated were: $y = 0.0031x - 0.0084$ for allantoin; $y = 0.0321x + 0.0275$ for

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uric acid; $y = 0.0246x - 0.0027$ for hypoxanthine; and $y = 0.0103x - 0.0921$ for creatinine. In all instances, the correlation coefficients were greater than 0.99. The standard addition method (standard plus urine) was used in determining chemical interferences of different analytes. The equations calculated were: $y = 0.0029x + 0.07436$ for allantoin; $y = 0.0324x + 0.4752$ for uric acid; $y = 0.0238x + 0.5110$ for hypoxanthine; and $y = 0.0098x + 0.6384$ for creatinine. The slopes for the calibration and standard addition graphs were similar for each of the four compounds. The detection limits were 2.91 mg/L for allantoin; 0.69 mg/L for uric acid; 0.53 mg/L for hypoxanthine; and 9.41 mg/L for creatinine with a 20 μL injection. The detection limit was determined from the calibration curves according to the method described by Miller and Miller.[13]

The analytical recovery was determined by triplicate analyses of urine samples spiked with standards of the metabolites at concentrations ranging from 10 to 50 mg/L for allantoin; 5 to 20 mg/L for uric acid; 5 to 25 mg/L for hypoxanthine; and 20 to 60 mg/L for creatinine. The main recovery was 97.15 ± 2.48 (RSD = 2.55%) for allantoin; 100.49 ± 3.85 (RSD = 3.83%) for

Table 1. Inter-day precision and accuracy.

Compound	Concentration added (mg/L)	Concentration found (mg/L; media ± SD; $n = 3$)	R.S.D (%)	Relative error (%)
Allantoin	10	9.85 ± 034	3.45	1.5
	20	18.96 ± 0.21	1.11	5.2
	30	30.25 ± 0.31	1.03	0.8
	40	38.31 ± 0.62	1.62	4.2
	50	47.91 ± 0.72	1.50	4.2
Uric acid	5	4.80 ± 0.09	1.80	4.0
	10	10.04 ± 0.10	1.00	0.4
	15	15.79 ± 0.06	0.35	5.3
	20	20.08 ± 0.06	0.28	0.4
Hypoxanthine	5	4.83 ± 0.01	0.21	3.4
	10	9.64 ± 0.28	2.91	3.6
	15	14.70 ± 0.04	0.28	2.0
	20	19.06 ± 0.03	0.16	4.7
	25	23.58 ± 0.05	0.21	5.7
Creatinine	20	19.66 ± 0.16	0.79	1.7
	30	28.50 ± 0.20	0.70	5.0
	40	40.80 ± 0.15	0.36	2.0
	50	51.45 ± 0.11	0.22	2.9
	60	58.50 ± 0.25	0.43	2.5

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uric acid; 96.13 ± 1.39 (RSD = 1.45%) for hypoxanthine; and 99.14 ± 3.27 (RSD = 3.30%) for creatinine. The interday precision and accuracy were assessed by analysing urine samples containing different concentrations of creatinine 5 times per day during one week. The results are given in Table 1.

CONCLUSION

The described ion-pair HPLC method has been shown to be linear, precise, and accurate. The final chromatographic conditions adopted (pH, methanol percentage, type of pairing-ion and concentration, column temperature, etc.) were a compromise between analyses time and resolution from any interfering substances. The urine sample preparation required only centrifugation and filtration before appropriate dilution.

Thus, this method is reliable for the simultaneous determination of creatinine and PD in urine samples, and we considered that this procedure may be proposed as a comparative method.

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Received April 1, 2003
Accepted April 30, 2003
Manuscript 6144

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