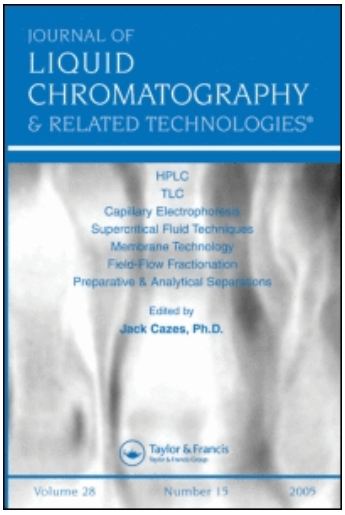

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Arín, M. J. , Díez, M. T. , Resines, J. A. and Alemany, M. T.(1990) 'Quantitative Estimation of Urinary Metabolites in Ruminants by High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 13: 12, 2465 — 2473
To link to this Article: DOI: 10.1080/01483919008049046
URL: http://dx.doi.org/10.1080/01483919008049046

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(12), 2465-2473 (1990)

QUANTITATIVE ESTIMATION OF URINARY METABOLITES IN RUMINANTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reversed-phase high-performance liquid chromatographyc method for a simultaneous determination of cretinine (C); Uric Acid (A); Hipoxanthine (HX); Xanthine (X); Hippuric Acid (HA); Benzoic Acid (BA) and Phenylacetic Acid (PAA) in urine of ruminants is described.

Separation was achieved on a Nova-PaK column by gradient elution. Quantitation and detection limits were determined Detection is effected by UV absorption at 254 nm with a total analysis time of less than 30 min. An aliquot of diluted urine is injected directly into the liquid chromatographic column.

The proposed HPLC method was verified for linearity, accuracy, precision and applicability.

INTRODUCTION

A series of compounds presents in the urinary excretion of ruminants were proposed recently as index of their nutritive status (1) (2), to stablish

relationship between energy and N intake, requirements and reserves disponibility in each phase of productive cycle.

In order to study these relationships, an analytical method is needed to determine the urinary metabolites. A series of papers (3-8) reported HPLC techniques for determination of some metabolites in biological fluids, have been published over the last few years.

This report described a rapid HPLC method for the simultaneously determination of seven metabolites presents in the sheep urine to stablish profiles and mathematical relationships which allow to define the nutritive status.

MATERIAL AND METHODS

Instruments

The analyses were performed on a Waters 600E equipped with a Waters 484, UV Detector. Separation was acomplished on a Nova-PaK C18 (3.9 x 150 mm) and the quantitation was based on integration of peak areas using a Waters 745B Integrator.

Chemicals

All reagents were Analytical Reagent Grade. Standards were purchased from Merck and used without further purification.

Methanol, HPLC grade, was purchased from Carlo Erba. Water was previously distilled and purified with a Milli-RO 15 Reagent Grade Water System (Millipore).

Urine samples

Urine samples were centrifuged and filtered through Millipore (0.45 µm) filter and diluted 10-fold (or more when the concentrations were high in samples) in distilled water. 20 µl of filtrate were injected directly to the column. The urine samples were stable for several weeks when stored at -20°C.

Standard Solutions

To prepare the standard curves, eight concentrations of standard solutions were made up in a range of concentrations from (10 to 100 µg/ml) for C; (2 to 100 µg/ml) for UA; (1 to 10 µg/ml) for HX; (2 to 10 µg/ml) for X; (10 to 100 µg/ml) for HA; (10 to 100 µg/ml) for BA and (50 to 200 µg/ml) for PAA. The stock solutions were stable when stored at 4°C until used.

The quantification were achieved by regression analysis of the peak areas of each analyte against concentration. Triplicate injections of each concentration were made.

Chromatographic conditions

HPLC separations were carried out using a 30 min linear gradient programmed from solvent A (0.025M aqueous sodium acetate, buffered to pH=4.5) to a mixture solvent A: methanol (75:25), v/v). The flow rate was maintained at 0.5 ml/min until the first 10 min and then increased to 1 ml/min until the end of analysis.

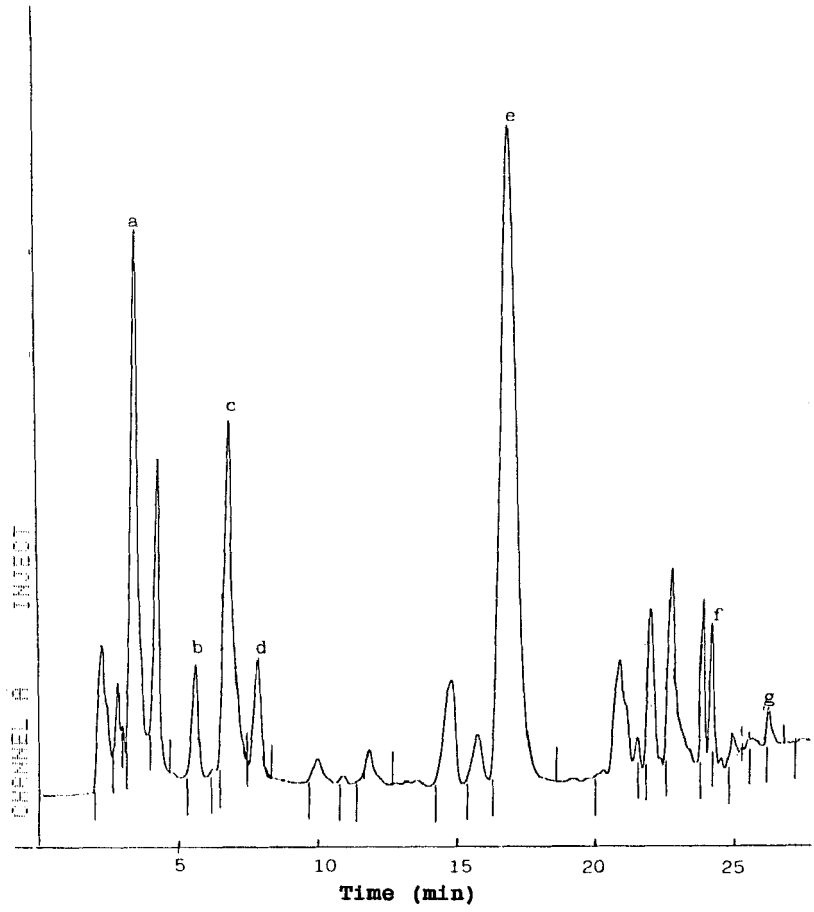

FIGURE 1.-Chromatogram of a ten-fold dilluted sheep urine. Peaks: a = C; b= UA; c = HX; d =X; e = HA; f = BA and g = PAA.

Detector was set at 254 nm.

Sample volumes were 20 µl, which were injected into the column through a Model U6K Injector (Waters Assoc.).

All analyses were carried out at room temperature.

The purity of the compounds peaks was tested by comparison of the peak areas obtained at wavelengths

of 245 and 254 nm from aqueous standards with those of various samples.

RESULTS AND DISCUSSION

Representative chromatogram obtained for urine samples is shown in Fig-1.

The retention times for C; UA; HX; X; HA; BA and PAA were approximately: 3.5; 5.4; 6.5; 7.3; 17.0; 23.8 and 26.0 min, respectively.

Calibration plots were linear over the concentrations range studied for the seven compounds. In all cases the correlation coefficients were found to be greater than 0.99. Fig-2 shows a typical calibration curve for HX.

The standard addition method was used to check for chemical interferences in the quantitation of different products. This method for HX in urine is shown in Fig-3. The slope found is similar to the calibration curve (Fig-2).

The limits of detection for ten-fold diluted urine were 1.0; 0.2; 0.1; 0.1; 0.7; 1.0; 7.0 µg/ml for C; UA; HX; X; HA; BA and PAA whith a 20 µl/ml injection.

The absolute recoveries of the seven analytes from urine were assesed by comparing peak areas obtained for the standard stock solutions of the analytes and urine spiked with the respective analytes. In all cases, the value was estimated by substracting the peak area obtained before spiking from the one found for spiked urine samples. Recoveries are shown in table I.

The interday precision, accuracy and reproducibility of the method over the entire concentrations range were determined whith the analysis of spiked urine samples. As shown in table I, the

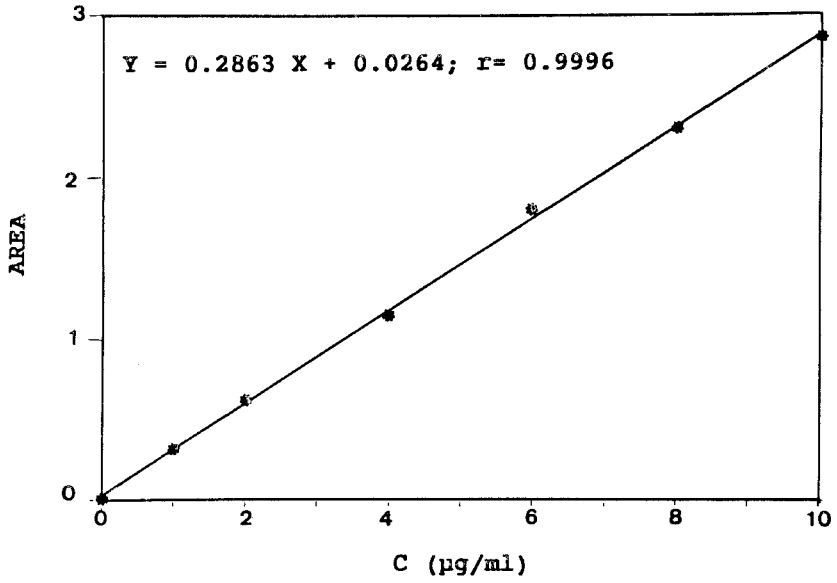

FIGURE 2.-Hipoxanthine calibration curve.

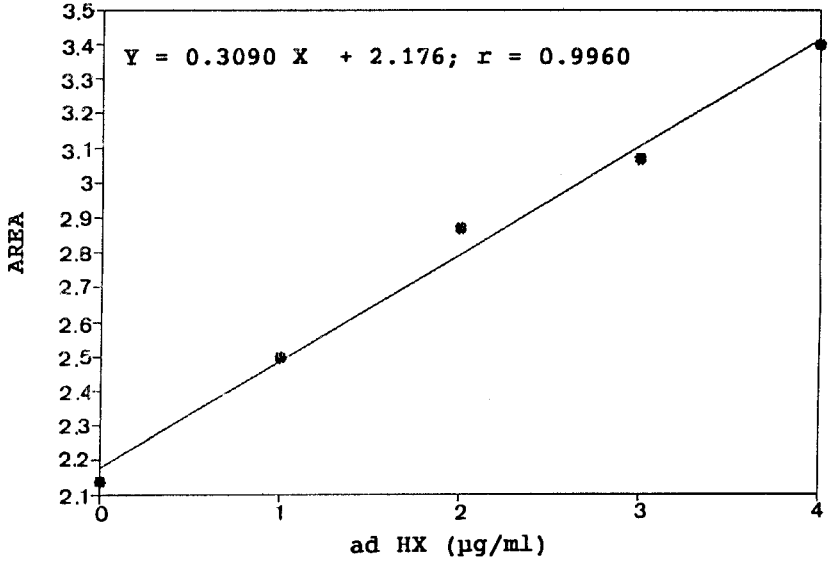

FIGURE 3.-Standard addition method for Hipoxanthine in sheep urine.

TABLE 1

Precision, Accuracy and Recoveries in the Determination of Metabolites in Sheep Urine

Comp. n=5	Conc. Added. (µg/ml)	Conc. found (µg/ml)	CV* (%)	RE** (%)	Recoveries Mean ± SD (%)
Creatinine	10	10.87 ± 0.34	3.2	8.7	100.44 ± 5.68
	20	19.73 ± 0.43	2.2	1.4	
	30	29.37 ± 0.89	3.0	2.1	
	40	38.60 ± 1.28	3.3	3.5	
Uric Acid	2	2.03 ± 0.04	2.0	1.5	101.24 ± 2.03
	3	3.08 ± 0.10	3.2	2.7	
	4	4.01 ± 0.02	0.5	0.2	
	5	5.03 ± 0.03	0.6	0.6	
Hipoxanthine	1	0.99 ± 0.02	1.6	1.0	100.18 ± 1.71
	2	2.02 ± 0.04	2.0	1.0	
	3	3.01 ± 0.04	1.5	0.3	
	4	4.02 ± 0.06	1.5	0.5	
Xanthine	1	0.98 ± 0.03	2.8	2.0	99.95 ± 1.87
	2	2.01 ± 0.03	1.5	0.5	
	3	3.00 ± 0.05	1.6	0.1	
	4	4.02 ± 0.05	1.3	0.5	
Hippuric Acid	10	10.42 ± 0.46	4.4	4.2	102.21 ± 3.70
	20	20.85 ± 0.83	3.9	4.2	
	30	30.23 ± 0.44	1.4	0.8	
	40	39.82 ± 0.48	1.2	0.4	
Benzoic Acid	20	20.85 ± 0.86	4.2	4.2	101.22 ± 3.10
	30	29.83 ± 0.80	2.7	0.6	
	40	40.46 ± 0.59	1.5	1.2	
	50	50.08 ± 0.58	1.2	0.2	
Phenyl-acetic acid	150	147.99 ± 2.73	1.8	1.3	99.50 ± 1.50
	300	303.36 ± 3.66	1.2	1.1	
	450	446.94 ± 6.66	1.5	0.7	
	600	593.52 ± 8.75	1.5	1.1	

* CV: Coefficient of variation; ** RE: Relative error

precision is expressed as the coefficient of variation and the accuracy as the relative error. For each analyte, interday precision was determined by assaying in 5 occasions, diluted urine samples spiked at 4 concentrations. In all instances, the coefficient of variation of peak area was found to be acceptable.

The accuracy was assesed by analysing known amounts of analytes. The observed concentrations were in good agreement with the actual concentrations.

In conclusion, we have developed a sensitive HPLC assay for the specific determination of Creatinine; Uric Acid; Hipoxanthine; Xanthine; Hippuric Acid; Benzoic Acid and Phenylacetic Acid in sheep urine, with high precision and accuracy. The method is also simple and rapid and suitable for routine analysis in clinical laboratories.

ACKNOWLEDGMENTS

This study was supported by a grant from C.I.C.Y.T. (GAN88/0071)

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