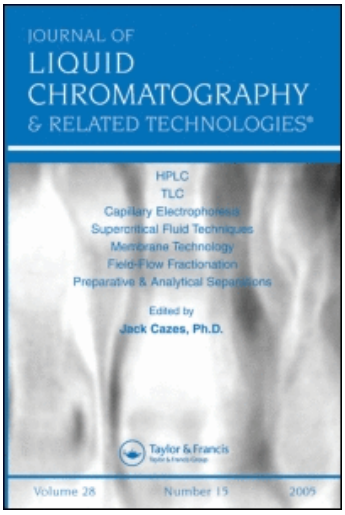

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SIMULTANEOUS DETERMINATION OF ALLANTOIN AND CREATININE IN URINE BY A RAPID REVERSED-PHASE LIQUID-CHROMATOGRAPHIC METHOD

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ABSTRACT

A simple, rapid, reproducible RP-HPLC method for a simultaneous analysis of allantoin and creatinine in sheep urine, is described.
Separation was achieved on a Novapak-C18 (3.9x300 mm) column by isocratic elution. Quantitation and detection limits were determined. Detection was effected at 218 nm. Retention times of allantoin and creatinine were 4.4 and 5.8 min, respectively.
The proposed RP-HPLC method was verified for linearity, accuracy, precision and applicability.

INTRODUCTION

Creatinine and allantoin are products presents in the urinary excretion of the ruminants that were proposed as index of their nutritive status in each phase of productive cycle, (1) (2).

Studies were realized to establish relationship between the urinary excretion of allantoin and creatinine and the amount of energy consumed by several ruminants,(3). Lindberg,(4), proposed the possibility of using creatinine as a internal marker to predict urinary allantoin excretion.

Energy is stored in the muscles in the form of phosphocreatine. When there is a need for energy in the body, the phosphocreatine is converted to creatine which is transformed to creatinine.

According to Brody,(5), creatinine is excreted in proportion to live weight within a wide range of body weights. Creatinine could be useful as an internal marker to make quantitative predictions of metabolic processes in intact animals,(4).

Allantoin is one of the end purine degradation products in the urine of ruminants,(6), and appears to be quantitatively the most important.

The urinary excretion of allantoin appears to correlate well with the intake of digestible dry matter and organic matter in ruminants,(4)(7)(8). Another researches gives further support to the usefulness of allantoin excretion in urine as indicator of microbial nucleic acids flow to the ruminants small intestine,(9)(10).

Various analytical methods have been described for the determination of allantoin and creatinine in biological fluids. Most procedures that have been published are based on colorimetric reactions.

The method most widely employed for the determination of creatinine is a Jaffe alkaline-picrate procedure, (11)(12). However the lower specificity of this assay is well known,(13)(14).

The traditional colorimetric analysis of allantoin in urine is based on the Rimini-Schryver reaction

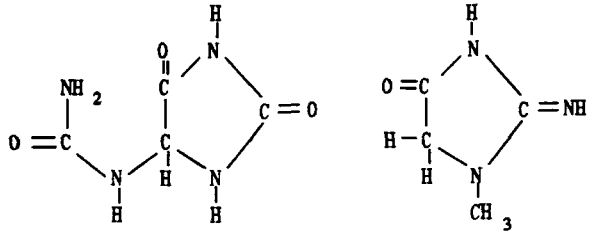

FIGURE 1- Chemical structures of allantoin and creatinine.

described by Young and Conway,(15). Lindberg,(16) and Chen,(17), described a method that adapted this reaction to the Technicon Autoanalyzer.

In the last years, several methods for the determination of creatinine, (18)(19)(20) and allantoin, (21), by HPLC in biological fluids are also described. Creatinine and allantoin are compounds with similar polarity, Fig-1. For this reason it is very difficult to separate them in such systems. The reverse-phase technique even though it may appear less specific, is generally considered more versatile than other methods.

This paper described a RP-HPLC method for the simultaneous determination of allantoin and creatinine in sheep urine.

Column temperature, pH, molarity and flow rate of the mobile phase, were optimized for maximum resolution, precision and minimum analysis time.

MATERIAL AND METHODS

Reagents

Allantoin was obtained from Sigma Chemical Co. and Creatinine were purchased from Merck and used without

further purification. Another chemicals were of the highest purity commercially available. Methanol was of HPLC-grade, obtained from Carlo Erba. Water was previously distilled and purified with a Milli-RO 15 Reagent Grade Water System (Millipore).

Standard Solutions

Creatinine and allantoin stock solutions, (1mg/ml), were prepared dissolving pure standards in water and were stored at 4°C for a month. Weekly, we prepared working standard solutions , 40 and 60 µg/ml respectively, by diluting the stock solutions with water; a 20µl aliquot of this solution was used daily as a control to check retention time and all other conditions of the HPLC procedure. A series of creatinine and allantoin working standards were made by dilution of the stock solutions with water.

Calibration curves were prepared over the concentration range 20 to 400µg/ml for the allantoin and 10 to 200µg/ml for the creatinine.

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

pH of mobile phase was obtained with potassium phosphate buffer in the range from 3.2-4.0 and acetate buffer for pH 4.5.

Urine Samples

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

Instruments

HPLC analysis were performed with a Waters 600E equipped with a Waters 484, UV Detector. Quantitation was based on integration of peak areas using a Waters 745B Integrator.

Chromatographic Conditions

A Novapak C18 reversed-phase column [30cm x 3.9mm (i.d), 4µm particles] was used. The mobile phase consisted of 10mM potassium phosphate buffer (pH 4.0). Before use, the mobile phase was always filtered through 0.45µm pore size (Millipore) and further degassed by sonication.

The flow rate was 0.5ml/min and the retention times were 4.4 and 5.8 min. for allantoin and creatinine respectively.

The column was maintained at 25°C. The absorbance detector was set at 218nm.

Creatinine and allantoin peaks were identified by their retention times and coelution with the authentic standards and quantized by comparing the peak areas of samples with those of authentic standards.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

Creatinine and allantoin are constituents of urine excretion of ruminants with a similar polarity. It is very difficult to separate these compounds in biological fluids.

We have studied, then, different procedures for obtaining a suitable separation with RP-HPLC under isocratic conditions and we tested several variables to optimize the separation of the creatinine peak from the

allantoin peak and from that other endogenous urinary compounds.

First, we have used a long column (30cm) to increase the theoretical plates. Then, we have varied the conditions of the eluent (pH, molarity, flow rate) and column temperature, in order to obtain a good resolution.

Finally, we had to find an optical wavelength for the detection. We studied the spectral behavior of allantoin and creatinine in the HPLC mobile phase over the wavelength range of 200-300nm. Although the maximum absorbance was 200nm for both compounds, we preferred to perform the analyses at 218nm, where the baseline was more stable.

Fig-2 (A-B) shows the modifications of allantoin and creatinine k'as the pH and molarity of the mobile phase, ranges from 3.2 to 4.5 and 4.0 to 10.0mM respectively. We preferred to adopt phosphate buffer, pH 4.0, because the use of acetate buffer, pH 4.5, gave deteriorated peak shapes at this wavelength.

k' did not show appreciable variations with the buffer solution molarity.

As Fig-2 (C-D) shows, the resolution decrease with the flow rate of mobile phase and with the column temperature.

On the basis of these results we decided to select the chromatographic conditions reported previously.

Because of the good reproducibility on retention time and peak areas and because no extraction was involved, an internal standard was omitted with consequent reduction in analysis time.

Fig-3 (A-B) shows chromatograms of the analyses of pure allantoin and creatinine standards and of urine under the chromatographic conditions finally adopted.

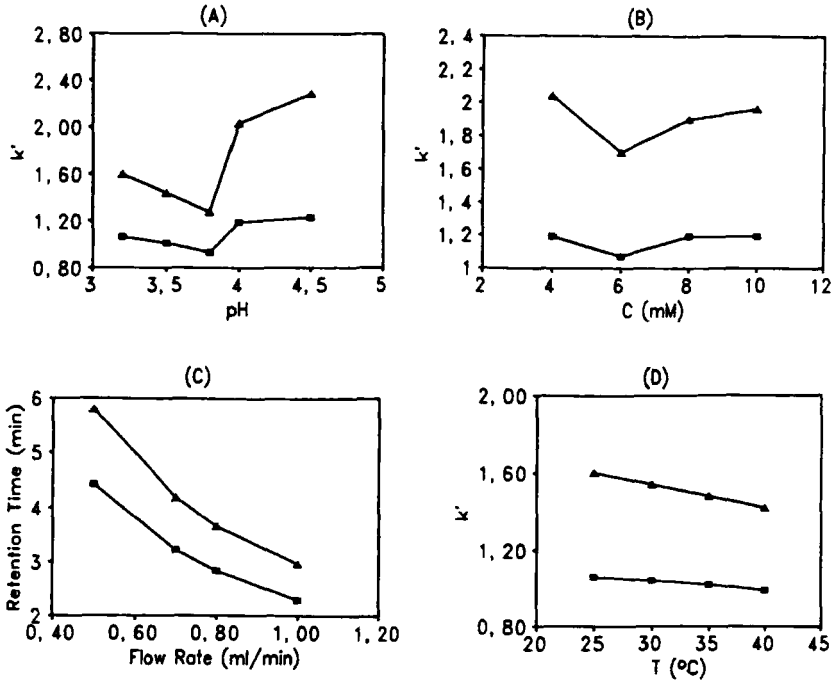

FIGURE 2- Optimization of the analytical conditions. (A)- Influence of mobile phase pH on the capacity factor (k') of allantoin and creatinine. (B)-Allantoin and creatinine (k') dependence on the mobile phase molarity. Mobile phase was potassium phosphate buffer, pH 4.0.(C)-Relation between allantoin and creatinine retention time and flow rate. Mobile phase was potassium phosphate buffer, 10mM. pH 4.0. (D)-Influence of column temperature on the capacity factor (k') of urinary metabolites. Allantoin (■) , Creatinine (▲).

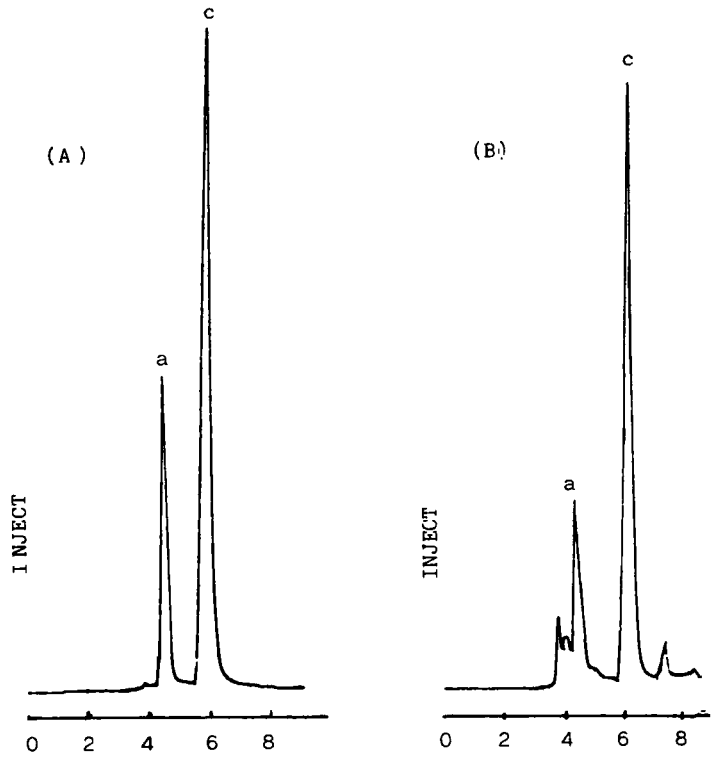

FIGURE 3- Chromatographic separation. (A)-Allantoin (60µg/ml) and Creatinine (40µg/ml) standard solutions. (B)-Urine sample.The arrow indicates the time of injection. HPLC conditions: Novapak-C18 [30cm x 3.9mm (i.d), 4µm particles]; mobile phase potassium phosphate buffer (10mM, pH 4.0); flow rate 0.5ml/min; column temperature 25°C; detector wavelength 218nm. Allantoin (a), Creatinine (c).

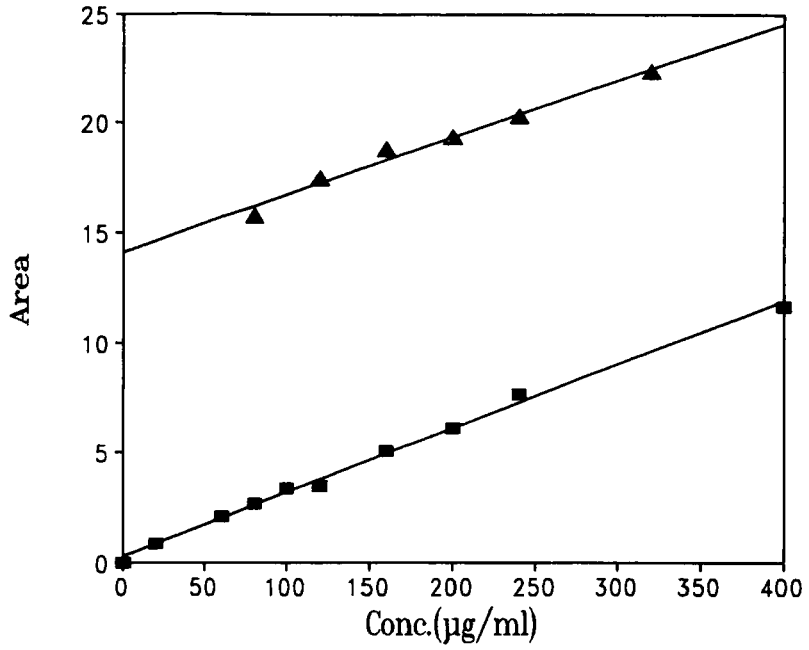

FIGURE 4- Calibration curve (■) and standard addition method (▲) for allantoin in sheep urine.

Analytical Variables

Linearity

A linear relationship between the peak area and the allantoin and creatinine concentrations in urine was obtained for the ranges of concentrations tested (20-400)µg/ml for allantoin and (10-200)µg/ml for creatinine. The equations calculated for allantoin and creatinine were, [y = 0.029x + 0.263] and [y = 0.167x + 0.844], respectively.

In both cases the correlation coefficients were found to be greater than 0.99.

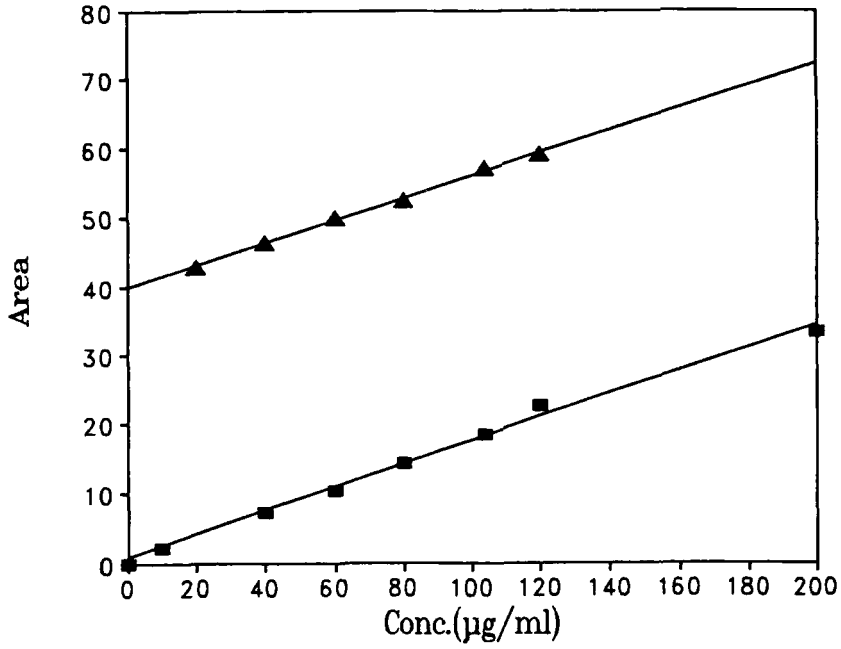

FIGURE 5- Calibration curve (■) and standard addition method (▲) for creatinine in sheep urine.

The standard addition method was used to check for chemical interferences in the quantitation of different products.

Fig-4 and Fig-5 show the calibration curves prepared by the addition of allantoin and creatinine to water and to samples. The slopes found were similar for each compound. The equations calculated in the standard addition method in urine were, [y = 0.026x + 14.091] and [y = 0.162x + 3.992], for allantoin and creatinine respectively.

Detection Limit

At a signal to noise ratio of 3, the detection limits of the method in urine were 1.0µg/ml for allantoin and 0.5µg/ml for creatinine.

Analytical Recovery

Recovery was determined by analysis of urine samples spiked with standards of allantoin and creatinine at concentrations ranging from (80-320)µg/ml and (20-120)µg/ml, respectively.

The recovery of added allantoin was 97.9 ± 1.7,(CV=1.8%) and 99.1 ± 0.6, (CV=0.6%) for creatinine.

Precision and Accuracy

Interday precision, accuracy and reproducibility of the method were assessed by analyzing three times per day for 10 days, three urine pools with different allantoin and creatinine concentrations.

Interday CV were 2.5%, 1.6% and 1.1% for the 40, 80 and 120µg/ml for creatinine and 2.2%, 1.8% and 1.2% for the 80, 120 and 160 µg/ml for allantoin.

In conclusion, we have developed a sensitive HPLC assay for the specific determination of allantoin and creatinine with high precision and accuracy.

The final chromatographic conditions adopted were a compromise between analysis time, peak shape and symmetry, and resolution from allantoin and creatinine and for other interfering substances.

We consider this method suitable for proposal as a possible reference method.

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