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Rapid and simple method for the determination of urinary benzoic and phenylacetic acids and their glycine conjugates in ruminants by reversed-phase high-performance liquid chromatography

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ABSTRACT

A simple, rapid and reproducible reversed-phase high-performance liquid chromatographic method for the simultaneous determination of benzoic acid (BA), phenylacetic acid (PAA) and their respective glycine conjugates hippuric acid (HA) and phenaceturic acid (PA) in sheep urine is described. The procedure involves only direct injection of a diluted urine sample, thus obviating the need for an extraction step or an internal standard. The compounds were separated on a Nova-Pak C_{18} column with isocratic elution with acetate buffer (25 m*M*, pH 4.5)–methanol (95:5). A flow-rate of 1.0 ml/min, a column temperature of 35°C and detection at 230 nm were employed. These conditions were optimized by investigating the effects of pH, molarity, methanol concentration in the mobile phase and column temperature on the resolution of the metabolites. The total analysis time was less than 15 min per sample. At a signal-to-noise ratio of 3 the detection limits for ten-fold diluted urine were 1.0 μg/ml for BA and HA and 5.0 μg/ml for PAA and PA with a 20-μl injection.

INTRODUCTION

Ruminants have long been known to excrete large amounts of aromatic acids in their urine. The principal aromatic acids excreted are hippuric acid (HA) and phenaceturic acid (PA), which are the respective glycine conjugates of benzoic acid (BA) and phenylacetic acid (PAA). The amounts of these acids excreted by sheep and their dietary origin have been studied by Martin [1,2]. The urinary excretion of BA and HA has been correlated with the cell wall digestibility. Urinary HA excretion may reflect, to some extent, changes in cell wall digestibility due to variation in degradable protein supply [3]. Urinary PAA excretion is derived from rumen catabolism of phenylalanine [1].

In order to establish the relationship between the urinary excretion of these aromatic compounds and the contribution of dietary constituents, it is necessary to develop a simple and accurate method for the determination of these com-

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pounds in sheep urine. Several workers have proposed methods for the determination of urinary aromatic acids. Some procedures are based on colorimetric reactions and chromatographic methods. For BA determination, reversed-phase high-performance liquid chromatographic (RP-HPLC) methods have been most widely employed [4–6]. Various methods, including spectrophotometric [7], gas chromatographic (GC) [8,9], GC–mass spectrometric [10,11] and HPLC methods [5,12–14], have been used for the determination of free PAA in urine. Some of these methods are very sensitive but they required extraction procedures, expensive equipment or rather tedious manipulations and they have not been used routinely. As a method for the determination of HA, spectrophotometry after reaction with benzenesulphonyl chloride [15–18] was once widely accepted, but has gradually been replaced by GC [15,19] and HPCL [4,5,20–23] methods, because of their possible higher specificity. Comparative studies of spectrophotometric and chromatographic methods [15–17] show good agreement, but the results obtained by spectrophotometry were higher than those given by the chromatographic techniques. We have found no report of the determination of PA.

We have developed a simple and rapid RP-HPLC method for the simultaneous determination of BA, PAA and their glycine conjugates HA and PA in sheep urine, which allows their urinary excretion profiles and their relationship with the nutritive status of ruminants to be established.

EXPERIMENTAL

Chemicals and reagents

BA, PAA and HA were obtained from Sigma (St. Louis, MO, USA). PA was synthesized in the Instituto de Química Orgánica "Juan de la Cierva" (CSIC) according to Ford's method [24] and characterized by IR and ^{1}H NMR spectrometry and elemental analysis.

Other chemicals were of the highest purity commercially available. Methanol was of HPLC grade from Carlo Erba (Milan, Italy). Water was previously distilled and purified with a Milli-RO 15 system (Millipore, Bedford, MA, USA).

Stock solutions of aromatic acids (1 mg/ml) were prepared in water and kept at 4°C up to one month. These stock solutions were diluted with deionized water to prepare working standard solutions of 20 μg/ml for BA and HA and 300 μg/ml for PAA and PA. A 20-μl aliquot of these solutions was used daily as a control to check all conditions of the HPLC procedure.

Calibration graphs obtained with different concentrations ranging from 1 to 50 μg/ml for BA, 10 to 300 μg/ml for HA and 100 to 500 μg/ml for PAA and PA were constructed by plotting the peak areas of each compound against concentration. Triplicate injections of each concentration were made.

Urine samples

Urine from unselected sheeps, with different body weights and diets, was collected daily under toluene in metabolic crates. After removing the toluene, the urine was stored at −20°C until analysed. Urine samples were centrifuged for 10 min at 2000 g and filtered through a Millex-HV 0.45-μm pore size filter (Millipore) and then diluted ten-fold with deionized water. The extent of dilution was based on the results of preliminary experiments. A 20-μl aliquot was injected on to the HPLC column.

Instruments and chromatographic conditions

A Waters Model (Milford, MA, USA) 600-E instrument equipped with a Waters Model 484 UV detector was used. Quantification was based on integration of peak areas using a Waters Model 745B integrator.

RP-HPLC separations were carried out on a Nova-Pak C_{18} column (150 mm × 3.9 mm I.D.; 4-μm particles) (Waters). The mobile phase was acetate buffer (25 m*M*, pH 4.5)–methanol (95:5). Before use, the mobile phase was always filtered through an HA 0.45-μm pore size filter (Millipore) and degassed by ultrasonication. The flow-rate and column temperature were 1.0 ml/min and 35°C, respectively. The detection wavelength was set at 230 nm. With all system components in place, the column was equilibrated by passing the mobile phase at a flow-rate of 1.0 ml/min for at

least 30 min or until a steady baseline was obtained.

The purity of the compound peaks was tested by comparison of the peak areas obtained at wavelengths of 218, 230 and 254 nm.

RESULTS AND DISCUSSION

Method performance

We studied different procedures to obtain a suitable separation by RP-HPLC under isocratic conditions and tested several variables to optimize the analysis of these aromatic acids. We varied the conditions of the eluent and the column temperature.

Fig. 1A shows the influence of methanol concentration (3–10%) on the retention of the compounds. The aqueous component of the mobile phase was buffered at pH 4.5 with a 25 m*M* acetate buffer. In all instances, decreasing the concentration of methanol increased the retention of the solutes. With a mobile phase containing 5% methanol the resolution of all of the peaks was greater than 1.5

The resolution was also optimized by investigating the effect of the pH of the mobile phase. Fig. 1B shows the variation of the capacity factors with pH values of 3.0, 3.2, 3.5, 4.0, 6.0 and 7.0 (25 m*M* phosphate buffer) and 4.5 (25 m*M* acetate buffer). For all compounds, the retention decreased with increasing pH because of deprotonation of the carboxylic groups. Variations in elution order as a function of pH may be attributed to differences in the pK_a values. For example, the pK_a values for BA, PAA and HA are 4.2, 4.3 and 3.8, respectively. No literature value for the pK_a of PA is available. Good separation and a reduction in the overall analysis time of the metabolites was achieved at pH 4.5.

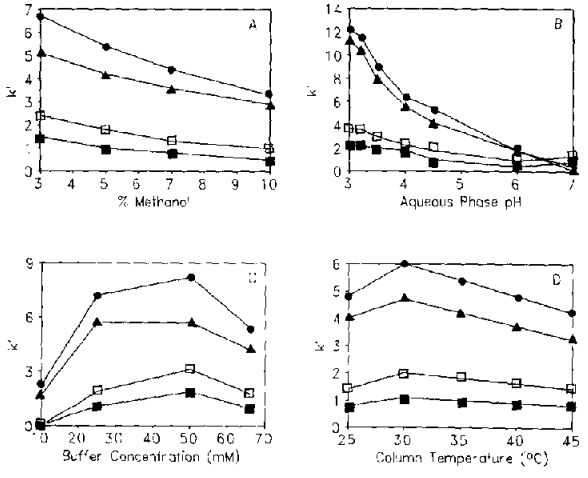

Fig. 1. Optimization of the analytical conditions. (A) Relationship between capacity factor (k') and methanol concentration. Aqueous phase was 25 m*M* acetate buffer (pH 4.5). (B) Dependence of k' on pH of the aqueous phase. The mobile phase contained 5% methanol. (C) Influence of the buffer concentration on k' values. Mobile phase was acetate buffer (pH 4.5)–methanol (95:5). (D) Effect of column temperature on k' values. Conditions as in (A), except that the mobile phase contained 5% of methanol. ▲ = BA; ● = PAA; ■ = HA; □ = PA.

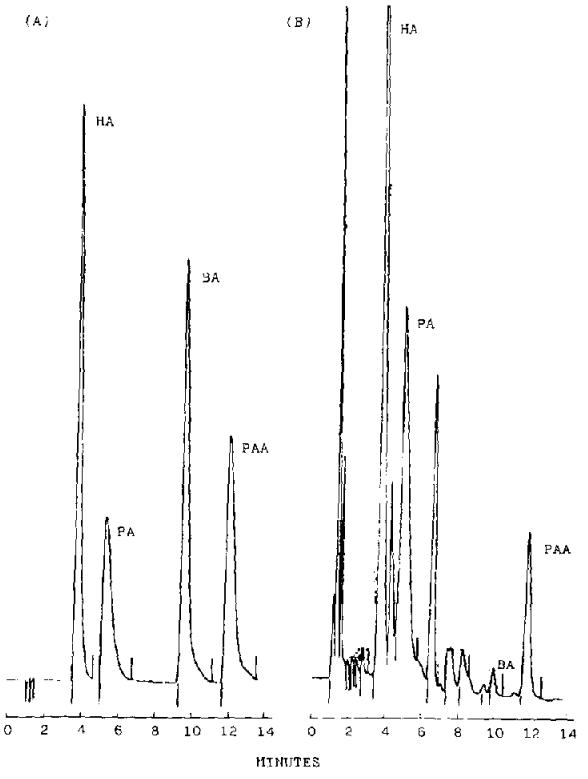

Fig. 2. Chromatographic separation. (A) Standard solution; (B) urine sample. HPLC conditions: Nova-Pak C_{18} column (150 mm × 3.9 mm I.D., 4-μm particles), mobile phase, acetate buffer (25 m*M*, pH 4.5)–methanol (95:5); flow-rate, 1.0 ml/min; column temperature, 35°C; detector wavelength, 230 nm.

The effects of buffer concentration (10–65 m*M*) and column temperature (25–45°C) on the capacity factors at this pH are shown in the Fig. 1C and D, respectively.

The studies described were performed at 35°C with a buffer concentration of 25 m*M* and at a flow-rate of 1.0 ml/min. Further increases in flow-rate (up to 1.5 ml/min) and temperature (up to 60°C) resulted in incomplete resolution of the compounds.

The detector wavelength was set at 230 nm, which is intermediate between the maximum absorbance of HA and PA (about 218 nm) and the BA and PAA (about 254 nm).

According to these studies, the best resolution for the simultaneous determination of these acids in sheep urine was obtained under the chromatographic conditions specified under Experimental.

The chromatograms resulting from the injection of pure standards and of ten-fold diluted urine under the chromatographic conditions finally adopted are presented in Fig. 2. Under these conditions there were no other endogenous urinary components that can interfere with the peaks of the analytes. The retention times were *ca.* 4.0 min for HA, 5.5 min for PA, 10.4 min for BA and 12.7 min for PAA. At a signal-to-noise ratio of 3, the detection limits for ten-fold diluted sheep urine were 1.0 μg/ml for BA and HA and 5.0 μg/ml for PAA and PA for a 20-μl injection.

Linearity

Linearity was checked by measuring different concentrations in the ranges 10–300 μg/ml for HA, 1–50 μg/ml for BA and 100–500 μg/ml for PAA and PA. Linear relationships between the peak areas and the concentrations tested were found. The average slopes and *y*-intercepts of the calibration graph equations are shown in Table I.

The standard addition method was used to check for chemical interferences in the determination of different acids. The equations calculated are shown in Table I.

The slopes found for the calibration and standard addition graphs were similar for each compound.

Analytical recovery, precision and accuracy

The analytical recovery was evaluated by assaying urine samples spiked with different amounts of each acid ranging from 10 to 50 μg/ml for BA and HA and from 100 to 500 μg/ml for

TABLE I

CALIBRATION GRAPHS AND STANDARD ADDITION METHOD

Acid	Linear regression equation[a]	r^2	n^b	Concentration[c] (μg/ml)
Calibration				
HA	$y = 1.0053x - 4.5534$	0.9995	13	10–300
PA	$y = 0.0266x - 0.1940$	0.9986	9	100–500
BA	$y = 0.9826x - 0.0190$	0.9998	9	1–50
PAA	$y = 0.0360x + 0.3833$	0.9989	9	100–500
Standard addition				
HA	$y = 1.0721x + 290.5432$	0.9986	5	10–50
PA	$y = 0.0241x + 4.0748$	0.9982	5	100–500
BA	$y = 0.9301x + 3.0239$	0.9998	5	10–50
PAA	$y = 0.0341x + 2.0006$	0.9980	5	100–500

[a] y = peak area × 10^{-5}; x = concentration (μg/ml).

[b] n = number of concentrations.

[c] Calibration, concentration range present; standard additions, concentration range added.

PAA and PA. Replicate analyses ($n = 5$) at each concentration were made. The mean recoveries were 101.27 ± 1.60% [coefficient of variation (C.V.) = 1.6%] for HA, 96.26 ± 2.77% (C.V. = 2.9%) for PA, 97.91 ± 2.64% (C.V. = 2.7%) for BA and 98.07 ± 2.45% (C.V. = 2.5%) for PAA.

The inter-day precision and accuracy were assessed by analysing urine samples containing different concentrations of each metabolite five times per day during one week. The results are given in Table II.

The quantitative data for BA, PAA, HA and PA obtained with this method are given in Table III. The mean values and standard deviations for BA, PAA and HA obtained from 40 urine samples were 23.50 ± 16.22, 312.83 ± 159.76 and 175.81 ± 92.30 μg/ml, respectively. The mean value for PA in 20 samples was 212.91 ± 169.61 μg/ml. In the other samples PA was not detected.

TABLE II

INTER-DAY PRECISION AND ACCURACY

Acid	Concentration added (μg/ml)	Concentration found (mean ± S.D; $n = 5$) (μg/ml)	C.V. (%)	Relative error (%)
HA	10	10.36 ± 0.17	1.6	3.6
	20	19.84 ± 0.52	2.6	0.8
	30	30.21 ± 0.79	2.6	0.7
	40	40.49 ± 0.85	2.1	1.2
	50	51.15 ± 1.33	2.6	2.3
PA	100	97.60 ± 1.76	1.8	2.4
	200	191.58 ± 13.03	6.8	4.2
	300	293.74 ± 6.46	2.2	2.1
	400	373.29 ± 16.42	4.4	6.6
	500	464.62 ± 22.30	4.8	7.1
BA	10	10.21 ± 0.57	5.6	2.1
	20	19.50 ± 0.55	2.8	2.5
	30	28.99 ± 1.19	4.1	3.3
	40	38.23 ± 1.34	3.5	4.4
	50	47.78 ± 1.67	3.5	4.4
PAA	100	100.46 ± 8.13	8.1	0.5
	200	193.48 ± 8.90	4.6	3.2
	300	296.79 ± 2.67	0.9	1.1
	400	393.83 ± 7.48	1.9	1.5
	500	469.23 ± 1.88	0.4	6.1

TABLE III

DETERMINATION OF HA, PA, BA AND PAA IN SHEEP URINE

Acid	n	Concentration (μg/ml)	
		Range	Mean ± S.D.
HA	40	18.00–296.60	175.81 ± 92.30
PA	20	14.46–396.27	21.91 ± 169.71
BA	40	4.20–49.76	23.50 ± 16.22
PAA	40	54.68–472.56	312.83 ± 159.76

The wide range of the values obtained is due to the variability between animals.

After injecting about 200 samples into the column, there were no appreciable changes in the pressure and the quality of the peaks was still acceptable.

CONCLUSION

An RP-HPLC system has been developed for the simultaneous separation of BA and PAA and their glycine conjugates HA and PA in sheep urine, in which no time-consuming extraction procedure is necessary, a simple mobile phase is used and good reproducibility is achieved. The method should be applicable to studies designed to reveal a better understanding of the mechanisms of aromatic acids metabolism in ruminants.

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