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Short communication

Improved HPLC method for the simultaneous determination of allantoin, uric acid and creatinine in cattle urine

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Abstract

An HPLC procedure developed for the rapid and simultaneous determination of purine derivatives (PD) in ruminants' urine was investigated, since the adoption of a single method for the simultaneous detection of PD and creatinine was not carried out due to elution of polar co-extractives and also due to overlapping of the peaks of allantoin and creatinine. The experimental conditions chosen in the present study avoid the presence of chemically competitive compounds and afford a good separation of the peaks of allantoin and creatinine. The recoveries of the standard compounds added to urine samples were 94–104%. This method can be proposed as a possible reference method for the estimation of allantoin, uric acid and creatinine in cattle urine.

Keywords: Purine derivatives; Allantoin; Uric acid; Creatinine

1. Introduction

The purine derivatives (PD) in the urine of ruminants have been validated as an index of microbial protein production [1,2]. PD concentration in urine can be estimated by using both the colorimetric and HPLC methods [3]. Colorimetric methods need lower technology requirements, but when compared to HPLC techniques [4,5] these methods are less sensitive to small variations in PD concentrations and are vulnerable to matrix effects [6]. However, there exist a good correlation between the colorimetric and HPLC methods. The simultaneous determination of PD (allantoin, uric acid, xanthine and hypoxanthine) and creatinine using HPLC is difficult due to elution of polar co-extractives resulting in the overlapping of peaks [6]. Moreover, it is very difficult to separate the peaks of allantoin and creatinine in biological fluids due to similarity in chemical structure and this has led to the development of ion pair RP-HPLC methods [7,8]. However, analysis time is greater in these methods [7,8]. A simple and rapid method for the simultaneous determination of PD and creatinine in ruminants' urine has also been developed previously [9]. The difficulties mentioned above, limits the use of this method for the quantification of PD and creatinine. Since, salvageable PD (xanthine and hypoxanthine) are below the detectable levels in cattle urine [1], the present communication put on record on the various changes made in this method, which makes it useful for the simultaneous estimation of allantoin, uric acid and creatinine in cattle urine.

2. Experimental

The mixed standards and urine samples were analyzed both by the earlier method [9] and by the improved method. Modifications made are discussed below.

2.1. Instrumentation

A model 10 A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a UV spectrophotometric detector set at 220 nm was used.

2.2. Reagents

Allantoin and xanthine was purchased from Sigma (Steinheim, Germany). Uric acid was obtained from Lancaster (Morecambe, England), creatinine was purchased from Merck (Mumbai, India) and hypoxanthine was obtained from Fluka

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(Steinheim, Germany). Xanthine and hypoxanthine were used in the preliminary study in which urine samples were analyzed following the earlier method [9]. Other chemicals used in the experiment were HPLC grade water (Qualigens, Mumbai) and potassium dihydrogen phosphate (HiMedia, Mumbai).

2.3. Standard solution

The stock standard solutions (1 mg/mL) of allantoin and creatinine were freshly prepared in water. Uric acid standard was also dissolved in water (1 mg/mL) by adding 0.01N sodium hydroxide solution (5 mL/100 mL stock standard solution) to make the pH 7. Immediately after the uric acid standard was completely dissolved, its pH was adjusted to three using 0.01N sulphuric acid. A series of mixed working standards were prepared just before injection, by adjusting the pH to neutral with 0.01N sodium hydroxide and 0.01N sulphuric acid, and triplicate injections of 20 μL for each concentration were made. Calibration graphs were prepared over the concentration range of 50–400 μg/mL for allantoin, 25–200 μg/mL for creatinine and 5–35 μg/mL for uric acid.

2.4. Preparation of urine samples

Urine was collected from crossbred bulls in metabolic cages into plastic containers having 500 mL of 10% sulphuric acid (to maintain pH < 3). Twenty milliliters aliquots were preserved pending analysis at −20 °C. Just before analysis, the urine samples were centrifuged and filtered through a Millipore filter (0.22 μm pore size) and was diluted ten fold with water after adjusting the pH to 7 using 0.01N sodium hydroxide and 0.01N sulphuric acid.

2.5. Chromatographic conditions

The quantitative HPLC separations were performed at a temperature of 25 °C (controlled by an oven) on a Phenomenex C18 (Model Luna 5 μ C 18 (2); Spincotech, Madras) reversed-phase column (250 × 4.60 mm I.D., 5 μm particle size). The mobile phase was 10 mM potassium dihydrogen phosphate solution (pH 4.7). The flow rate was 1 mL/min and the absorbance detector was set at 220 nm.

2.6. Quantification of purine derivatives in urine sample

Compound peaks were identified by the retention times and quantified by comparison of the peak areas of the samples with those of authentic standards on a 20 μL injection.

3. Results and discussion

The chromatogram of the mixed standards and urine sample obtained by following the experimental conditions suggested by the earlier method (Table 1) [9] is shown in Figs. 1 and 2, respectively. This method [9] was unable to give separate peaks for allantoin and creatinine.

Earlier method [9] suggests the use of water for dissolving the standards. When stock standard solutions (1 mg/mL) were prepared, the standards of allantoin and creatinine dissolved completely in water while uric acid standard dissolved only by adding 0.01N sodium hydroxide solution (5 mL/100 mL stock standard solution). Immediately after the standards were completely dissolved, the pH of the standard solutions was brought to three using 0.01N sulphuric acid to prevent the degradation of PD at higher pH. Just before injection, the pH of the mixed

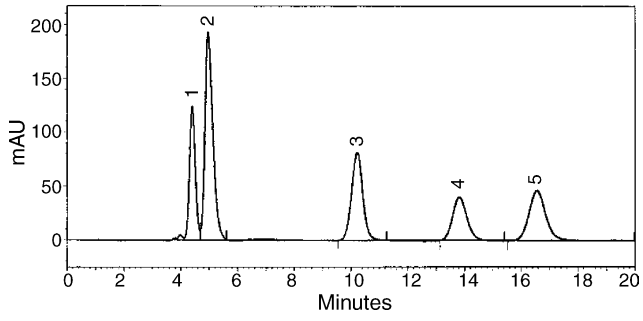

Fig. 1. Chromatogram of standard mixture analyzed following the earlier method [9]. The peaks and their retention times are: (1) allantoin at 4.4 min, (2) creatinine at 5.0 min, (3) uric acid at 10.2 min, (4) hypoxanthine at 13.8 min and (5) xanthine at 16.5 min.

Table 1
A summary of the characteristics of the earlier method and the improved method

Particulars	Characteristics	
	Earlier method [9]	Improved method
Conditions	Isocratic	Isocratic
Column	C18 reversed-phase (Novapak) (300 mm × 3.9 mm I.D., 4 μm)	C18 reversed-phase (Phenomenex) (250 mm × 4.60 mm I.D., 5 μm)
Mobile phase	10 mM potassium phosphate Buffer (pH 4.0)	10 mM potassium dihydrogen phosphate Solution (pH 4.7)
Flow rate	0.5 mL/min	1 mL/min
Wavelength	218 nm	220 nm
Standards dissolved in	Water	Allantoin and creatinine in water, uric acid in alkalinized water
Filter used for urine	0.45 μm (Millipore)	0.22 μm (Millipore)
pH of standard mixture	–	7
pH of diluted urine sample	–	7

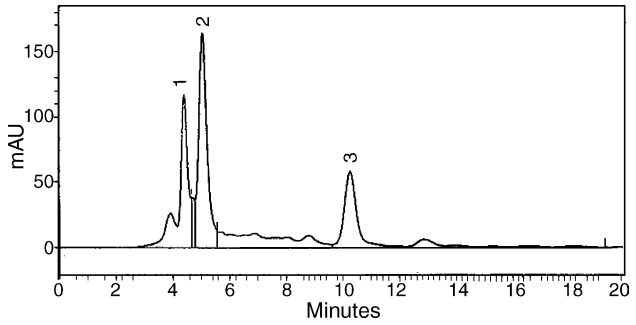

Fig. 2. Chromatogram of purine derivatives and creatinine in urine sample analyzed following the earlier method [9]. The peaks and their retention times are: (1) allantoin at 4.4 min, (2) creatinine at 5.0 min and (3) uric acid at 10.2 min.

standards and urine samples were brought to neutral because a pH of 7 provided a good separation of the peaks of allantoin and creatinine in urine samples and altered the retention time of the interfering compounds. The study revealed that, the retention time of creatinine is pH dependent and it increased with the increase in the pH of the standard/sample solution. However, the retention time of allantoin is pH independent and thus, a clear separation of the peaks of allantoin and creatinine can be obtained by increasing the pH of the standard mixture/urine sample to neutral (Figs. 3 and 4). Influence of pH of the standard mixture/urine sample on the retention time of analytes in a 20 μL injection is summarized in Table 2.

Earlier methods have diluted urine samples with water [9–11] or a weak buffer [4,8] taking no account of variations in urine pH. Variations in urine pH can alter the retention time of analytes in RP-HPLC analytical systems [12]. Moreover, it has been reported that urine with acidic pH can dissolve only about 1/10th as much total uric acid as that with pH 7 [13]. Urine samples are usually preserved at acidic pH for PD estimation in order to prevent the degradation of PD at alkaline conditions. Therefore, mere dilution of urine samples will not bring a pH of 7 and the existing methods may underestimate the uric acid content in urine. To prevent this, it is suggested to bring the pH of diluted urine to 7 just before injection.

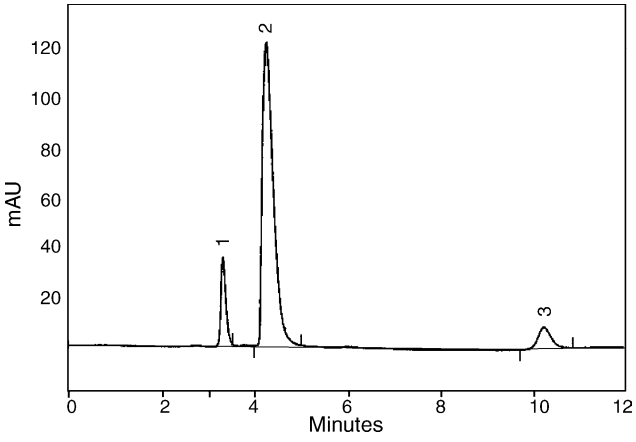

Fig. 3. Chromatogram of standard mixture analyzed following the improved method. The peaks and their retention times are: (1) allantoin at 3.3 min, (2) creatinine at 4.2 min and (3) uric acid at 10.2 min.

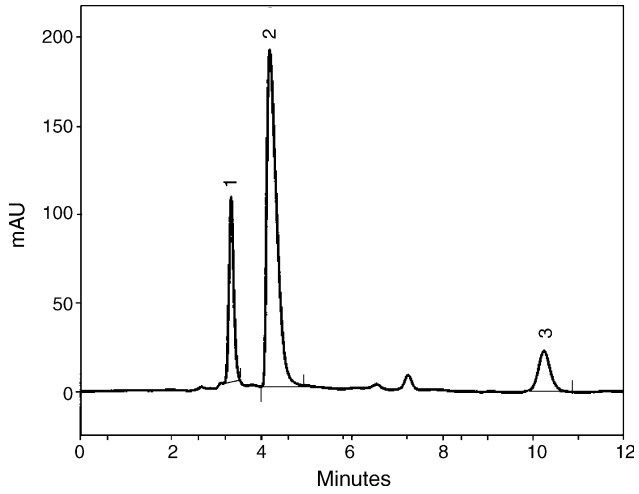

Fig. 4. The chromatogram of allantoin, uric acid and creatinine in urine sample analyzed following the improved method. The peaks and their retention times are: (1) allantoin at 3.3 min, (2) creatinine at 4.2 min and (3) uric acid at 10.2 min.

A linear relationship between the peak area and the concentration of standards in water was obtained and the equations calculated were $y = 0.0185x - 0.0199$ for allantoin, $y = 0.084x + 0.0103$ for creatinine and $y = 0.0885x + 0.0369$ for uric acid (y is the peak area $\times 10^{-6}$; x is the concentration). The standard addition method (standard plus urine) was used in determining chemical interferences of the analytes. The equations calculated were: $y = 0.0184x - 0.022$ for allantoin; $y = 0.083x + 0.1069$ for creatinine; and $y = 0.0912x + 0.0939$ for uric acid. The slopes for the calibration graphs for standards in water and in urine are statistically similar (t-Student test) for each of the three compounds. The accuracy of this improved method was assessed by examining the recovery of known quantities of allantoin, creatinine and uric acid added to urine samples. The recovery was determined by triplicate analysis of urine samples spiked with standards at concentration ranging from 2 to 150 μg/mL for allantoin; 0.5 to 40 μg/mL for creatinine and 0.2 to 15 μg/mL for uric acid. It can be seen that standards added to urine were recovered satisfactorily (94–104%) (Table 3). Mean recoveries of standards added to urine are consistent with the previously reported values [7–9]. The day-to-day precision of the method was obtained by triplicate analysis of seven aliquots

Table 2
Influence of pH of the standard mixture/urine sample on the retention time (RT) of analytes[a]

pH	RT in standard solution			RT in urine sample		
	Allantoin	Creatinine	Uric acid	Allantoin	Creatinine	Uric acid
3	3.3	3.5	10.1	3.3	3.5	10.1
4	3.3	3.8	10.1	3.3	3.8	10.1
5	3.3	4.0	10.1	3.3	4.0	10.1
6	3.3	4.1	10.2	3.3	4.1	10.2
7	3.3	4.2	10.2	3.3	4.2	10.2
8	3.3	4.2	10.2	3.3	4.2	10.2
9	3.3	4.2	10.3	3.3	4.2	10.3
10	3.3	4.2	10.3	3.3	4.2	10.4

[a] Retention time in minutes.

Table 3
Summarized results of recoveries (*R*, %) of standards added to cattle urine at different concentrations (*C*, μg/ml)[a]

	Allantoin	Creatinine	Uric acid
C	2	0.5	0.2
R	99.00 ± 0.65	100.00 ± 3.16	98.75 ± 3.75
C	5	1	1
R	97.80 ± 3.58	99.00 ± 4.45	98.25 ± 0.25
C	10	2	3
R	97.80 ± 3.02	100.50 ± 4.97	100.75 ± 0.50
C	20	5	5
R	99.51 ± 3.13	99.65 ± 1.44	104.25 ± 5.18
C	60	10	8
R	99.43 ± 0.72	94.00 ± 3.31	97.09 ± 0.90
C	100	20	10
R	98.90 ± 0.29	98.84 ± 1.07	99.75 ± 0.17
C	150	40	15
R	100.05 ± 0.74	99.00 ± 0.34	98.55 ± 1.59
R (Pooled data)	98.93 ± 0.74	98.7 ± 0.97	99.63 ± 0.94

[a] Compound recoveries (mean ± S.E.) determined from standard additions to 21 diluted urine samples.

Table 4
Precision of the method obtained in within-day ($n = 10$) and day-to-day ($n = 7$) measurements

Compound	Coefficient of variation (%)	
	Within-day	Day-to-day
Allantoin	3.36	3.73
Creatinine	3.48	4.02
Uric acid	6.17	6.25

of urine samples with various concentrations of standards on 7 consecutive days. Within assay coefficients of variations were calculated by repeatedly processing ($n = 10$) aliquots of spiked urine samples. The precision of the method obtained in within-day ($n = 10$) and day-to-day ($n = 7$) measurements is summarized in Table 4. The detection limits determined at a signal-to-noise ratio of 3 were 0.94 μg/mL for allantoin, 0.13 μg/mL for creatinine and 0.11 μg/mL for uric acid.

4. Conclusion

The improved HPLC method is effective in the simultaneous determination of allantoin, creatinine and uric acid in cattle urine, allowing a good separation between the peaks of allantoin and creatinine. The result obtained suggests that this method could be used suitably for simultaneous determination of purine derivatives and creatinine in cattle urine.

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