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Journal of Chromatography B, 744 (2000) 129–138

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantification of allantoin, uric acid, xanthine and hypoxanthine in ovine urine by high-performance liquid chromatography and photodiode array detection

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Received 12 November 1999; received in revised form 21 March 2000; accepted 7 April 2000

Abstract

A HPLC method for the determination of allantoin, uric acid, hypoxanthine and xanthine (purine metabolites) in ovine urine without the disadvantages inherent in derivatization is described. After dilution 1:6 with water, urine samples were injected onto the column. Separation and quantification of purine metabolites was achieved using two Nova-Pak C_{18} columns (4 μm, 250×4.6 mm, Waters). A binary gradient program and UV detection were used for purine metabolites analysis. Clear resolution of purine metabolites was obtained in less than 15 min. Allantoin, uric acid, hypoxanthine and xanthine in the effluent were monitored at 225, 284, 250 and 267 nm, respectively. The average recoveries of purine metabolite standards added to urine samples were satisfactory (100.2–102.9%). The low coefficients of variation (0.29–0.73%) as well as the low detection (0.16–0.70 nmol) and quantification (0.52–2.32 nmol) limits indicate satisfactory precision, reproducibility and sensitivity of the proposed method. This procedure is suitable for routine quantification of purine metabolites in a large number of urine samples.

Keywords: Allantoin; Uric acid; Xanthine; Hypoxanthine

1. Introduction

Quantification of rumen microbial synthesis is an important element in the estimation of nutritive value in ruminant feed evaluation systems [1–5]. A number of internal markers (purine metabolites, nucleic acids or 2,6-diaminopimelic acid) or exogenous isotopic markers (such as ^{15}N, ^{35}S or ^{32}P) [1] have been used to estimate the extent of microbial protein utilization by host animals [2,6–8].

Dietary-origin nucleic acids are degraded in the rumen [9] and the purines entering the duodenum appear to originate mainly from rumen microorganisms. Purines are metabolised in a series of reactions involving hypoxanthine, xanthine, uric acid and allantoin as end products that are subsequently excreted in urine [1,2,5]. Measurements of urinary excretion of purine metabolites, primarily allantoin or, additionally, uric acid, xanthine and hypoxanthine, has been proposed as a marker for microbial protein synthesis [10]. The major advantage of this method is that proposed approach does not require cannulated animals as do other methods, and the analysis of purine metabolites is relative simple. The problem of non-specificity of the colometric assay for purine metabolites [11] can be overcome by

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00239-5

using a multitude of methods with or without derivatization [2,8,12–25]. The main advantage of our recently proposed high-performance liquid chromatography (HPLC) method [14,15] is that urine samples are analyzed directly after dilution.

The combination of HPLC reversed-phase C_{18} columns with monitoring of the effluent at 205 nm provides an acceptable analytical tool for quantification of purine metabolites in the urine of ruminants. Detection at short UV wavelengths of up to 215 nm results in compromised method selectivity and therefore longer and more specific detection wavelengths were applied in the current method. In this study a combination of the direct separation and determination using a reversed-phase HPLC system equipped with a photodiode array detection (PAD) system was found to be accurate, more precise and selective than previously published methods for the determination of purine metabolites in urine.

2. Experimental

2.1. Reagents

Allantoin, uric acid, hypoxanthine and xanthine were obtained from Sigma (St. Louis, MO, USA). Methanol and other chemicals were of analytical reagent grade and purchased from POCh (Gliwice, Poland). Water used for the preparation of eluent buffers and for dissolving purine metabolites standards, urine samples, or chemical reagents was prepared using an Elix and Milli-Q system (Millipore, Toronto, Canada). The mobile phase (eluents A and B) was filtered through a 0.45-μm membrane filter (Millipore). Eluents A and B were degassed for 5–7 min under a vacuum with ultrasonication prior to use.

2.2. Chromatographic equipment

The instrument used consisted of a Waters 625LC system, which includes a controller for a gradient elution, and two Waters Model 501 pumps. The apparatus was coupled to a Waters 712 WISP autosampler, Water 996 PAD system and a computer data handling system (all equipment supplied by Waters, Millipore, MA, USA).

Purine metabolites in an effluent were monitored using PAD operated in a UV range from 201 to 288 nm with a spectral resolution of 1.2 nm and a measurement frequency of 2 spectra per second. Development of the analytical method, collection and data integration were performed using Millennium 2001 software and a Pentium 5P60 computer. The analytical columns used were two Nova-Pak C_{18} columns (4 μm, 250×4.6 mm, Waters) in conjunction with a guard column (Waters) of 10×6 mm containing reversed-phase C_{18} (30–40 μm) pellicular packing material.

2.3. Analytical solvents and gradient composition

A binary gradient program (Waters curvilinear program) was used for the complete separation of allantoin, uric acid, hypoxanthine and xanthine in ovine urine. The following elution solvents were used: solvent A was 0.0025 *M* $NH_4H_2PO_4$ buffered to pH 3.5 with 10% phosphoric acid. Solvent B was solvent A–methanol (95:5, v/v). The gradient composition is shown in Table 1. The maximum system pressure was 34.4 MPa. All separations were performed at a column temperature of 24°C, while the ambient temperature was 19–21°C. Injection volumes were 5, 10 or 20 μl.

Purine metabolites peaks were identified on the basis of the retention time of processed standards injected separately and by adding standard solution to urine samples. The values of purine metabolites retention times were based on mean±standard deviation (SD) of 35–37 urine samples. The limits of detection (LODs) were calculated at a signal-to-background ratio of 3, while the limits of quantification (LOQs) were defined as 10 times the background under a peak [23]. The background under the peak was calculated from the baseline from the left and right side of the peak.

2.4. Sample preparation

Urine was collected from sheep B in a metabolic cage and diluted 1:3 with HPLC-grade deionized water. Then the same series of urine samples was preserved with 100 μl of $CHCl_3$ added per liter of samples or by adding 1 *M* H_2SO_4 or 12 *M* HCl to reduce the pH to below 3. The preserved urine

Table 1
Gradient composition

Time (min)	Flow-rate (ml min^{-1})	Composition (%)	
		Solvent A	Solvent B
0	1	100	0
3.5	1	60	40 (linearly increased from 3 min)
11.5	1	20	80 (linearly increased from 3.5 min)
15.3	1.15[a]	20	80
15.4	1.15	0	100 (linearly increased from 15.3 min)
30.8	1.15	0	100
31.0	1.20	100	0 (linearly changed from 30.8 min)
53.0	1.20	100	0

[a] The flow-rate linearly increased from 14 min.

samples were stored at −20°C until analysis. For HPLC analysis urine samples were mixed and then again diluted with HPLC-grade deionized water so that the final dilution was no less than 1:6.

In order to validate the preservation procedure, a comparison between purine metabolites concentrations in fresh, non-preserved and preserved urine samples was conducted. Thus, to validate the urine collection and storage method, urine obtained from sheep A in a metabolic cage was diluted 1:6 with HPLC-grade deionized water. Then to the same series of urine samples an appropriate amount of preservation agent (preserved urine samples) or amount of deionized water (fresh or non-preserved urine samples) was added. The urine samples were stored at −20°C until HPLC analysis. All assayed urine samples stored at room temperature (19–21°C) were protected from light.

3. Results and discussion

The main analytical problem in the present study was to obtain suitable separation of purine metabolites peaks from background fluctuations and interfering species in urine samples. However, many components of urine samples possess a high polarity and/or a strong chromophore in the short UV wavelength range (195–215 nm), which are coincident with the short UV absorbances of purine metabolites (Fig. 1A). Obviously, these interfering species can reduce the specificity of purine metabolites monitoring (especially of allantoin) in this UV region [8,12]. On the other hand, purine metabolites can be satisfactorily separated using columns packed with silica-based bonded phase with strong hydrophobicity. Thus, in order to improve the specificity of the HPLC procedure, a reversed-phase C_{18} column containing dimethyloctadecylsilyl-bonded amorphous silica was applied, because these columns usually resulted in suitable separation of analytes of even weak hydrophobicity from aqueous solutions. Therefore, in order to improve the method specificity, especially for allantoin, two long C_{18} columns (Nova-Pak) together with UV detection at wavelengths >224 nm were applied. Really, allantoin monitored at 225 nm possess a relatively high absorbance (Fig. 1B), while other nearest interfering species have a lower absorbance in comparison with that at short wavelengths (i.e., <220 nm). Our studies documented that values of area ratios of the allantoin peak to peaks of the nearest interfering species depended upon the UV wavelengths used in monitoring (Table 2). Indeed, allantoin produced a smaller signal at 225 nm as compared with UV signals at <225 nm, however interfering species have negligible absorbance at 225 nm. Therefore, monitoring at 225 nm is a compromise between values of the allantoin peak and peaks of closely eluting impurities. As can be seen from a chromatographic run of a urine sample (Fig. 1B), the procedure resulted in suitable separation and quantification of allantoin, which eluted at 5.23±0.17 min (mean±SD of 35 samples). Unfortunately, unsatisfactory separation of purine metabolites, especially of allantoin, from interfering species was observed when a single column was used. Acceptable separation of uric acid, hypoxanthine and xanthine (oxy-

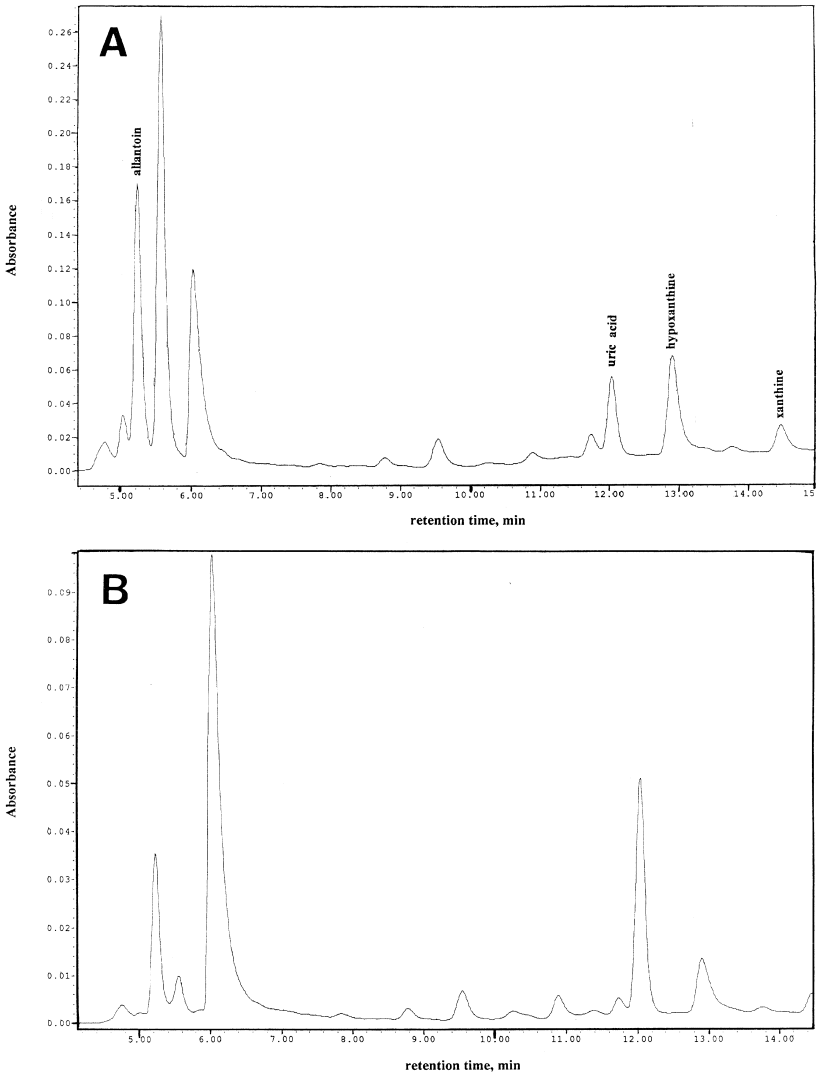

Fig. 1. Chromatograms for (A) urine sample using the current HPLC method (UV detection at 205 nm), (B) allantoin: 5.23±0.17 min (UV detection at 225 nm), (C) uric acid: 12.16±0.21 min (UV detection at 284 nm), (D) hypoxanthine: 12.86±0.19 min (UV detection at 250 nm), (E) xanthine: 14.38±0.21 min (UV detection at 267 nm).

purines) from background fluctuation and interfering species present in urine samples was obtained, however, by using two long C_{18} columns (Nova-Pak), the proposed gradient program (Table 1) and UV monitoring at longer wavelengths (i.e., at 284, 250 and 267 nm, respectively) (Fig. 1C–E). Moreover, high sensitivity of the oxypurines assay was achieved due to monitoring at the maximum of absorbances in the longer wavelength UV range (>245 nm).

As expected all purine metabolites peaks were absent from the blank when the proposed gradient program (Table 1) was used. Satisfactory separation of purine metabolites from unidentified species can be achieved using the slow concentration increase of solvent B containing a low level of methanol. After

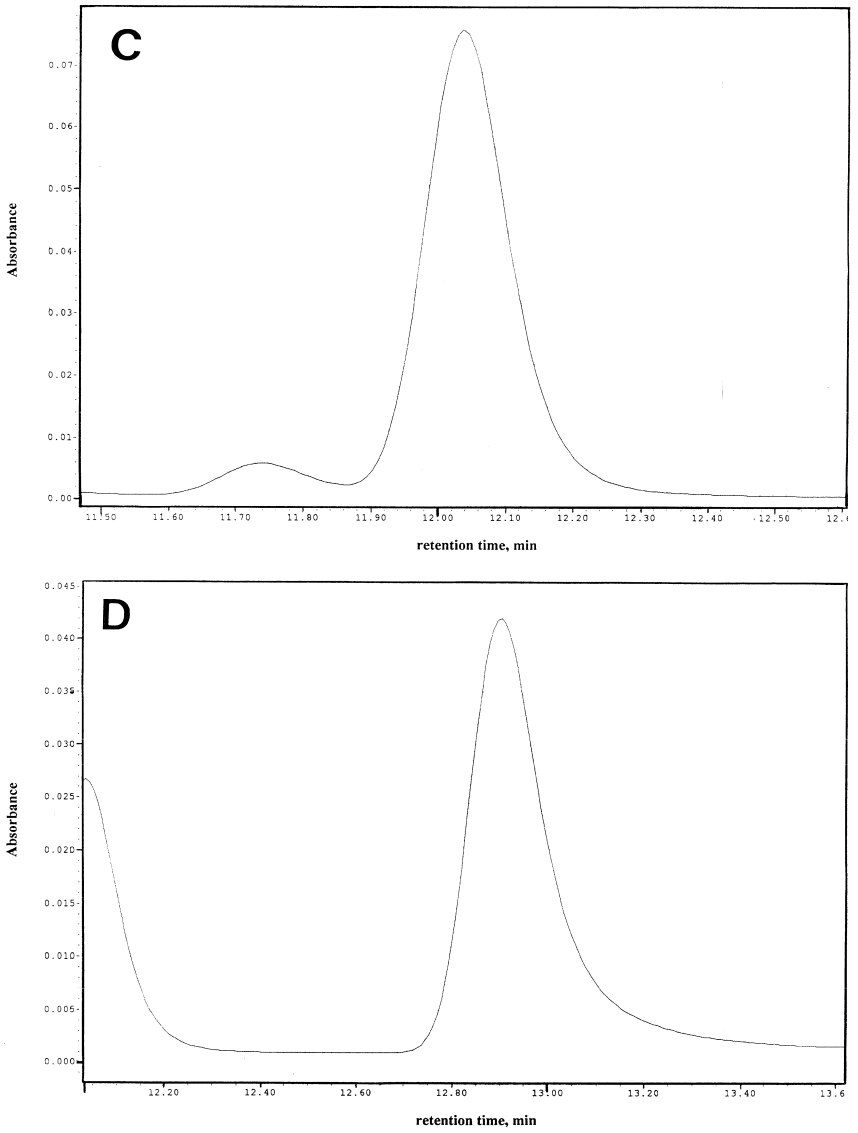

Fig. 1. (*continued*)

16 min columns were conditioned for 15 min (in 100% solution B) and then re-equilibrated for 22 min (in 100% solution A), since some strongly retained species were detected in the column effluent after 50 min of the analysis time. On the other hand, increase the methanol content in solvent B resulted in an increases in system pressure. Obviously, the high system pressure may be a serious disadvantage of this method used for routine analysis of a large number of urine samples.

The rapid and high increase of the methanol content in a mobile phase (near 60–100%, v/v) may result in precipitation of some species in the chromatographic system and should also be followed by long re-equilibration at 100% solution A.

The responses (a peak area, S_n) of purine metabo-

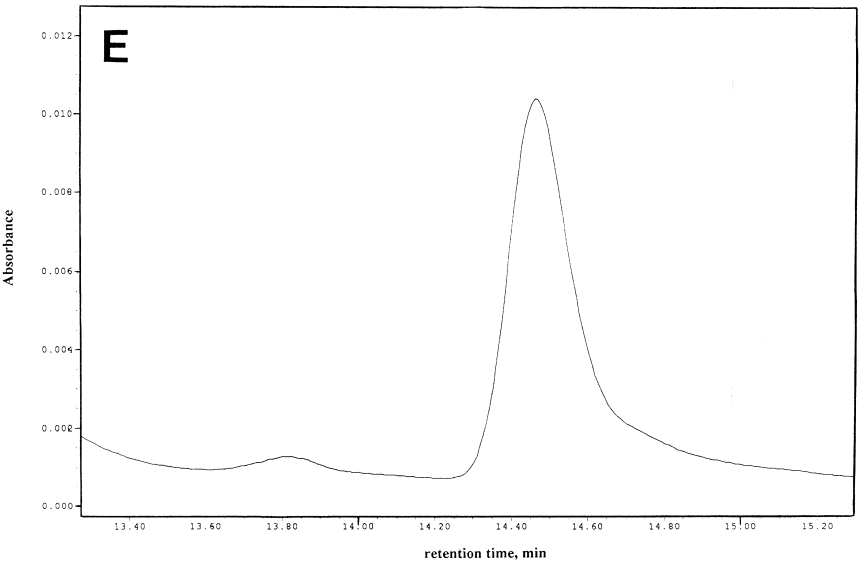

Fig. 1. (*continued*)

lites were linearly related to the amount of purine metabolites within a wide range of purine metabolites in standards and in urine samples (Table 3). As can be seen from the results summarized in Table 3, the proposed UV monitoring appears suitable (see the *r* and SES values) for purine metabolites assays; detection at the longer wavelengths offers better linearity of oxypurines as compared with monitoring at 205 nm.

The methods used to preserve urine from decomposition during collections from sheep or storage at 19–22°C until analysis were compared by examining the purine metabolites levels in urine preserved using $CHCl_3$, 1 *M* H_2SO_4 or 12 *M* HCl. As expected, in all of the preserved urine samples, the determined purine metabolites contents agreed fairly well (Table 4). Only allantoin concentrations in urine preserved with $CHCl_3$ were slightly lower in comparison with the control urine sample and urine preserved with HCl or H_2SO_4. The effect of storage time on purine metabolites contents showed that the concentrations of these metabolites in urine preserved with $CHCl_3$ were only negligibly changed even when samples were stored for 38 h at room temperature (sheep B).

In order to validate preservation procedures, a comparison between purine metabolites concentrations in non-preserved samples (urine storage time – 2 or 25 h) and preserved urine samples was performed. The results given in Table 4 (sheep A)

Table 2
The peak areas of allantoin (S_n, μV s) and the nearest unidentified species (peaks 1 and 2) in urine sample depending on the wavelengths of monitoring (injection volume was 10 μl)

Species	t_R [a] (min)	Wavelengths of UV monitoring						
		200 nm S_n	205 nm S_n	210 nm S_n	215 nm S_n	220 nm S_n	225 nm S_n	230 nm S_n
Peak 1	5.03	$103.0 \cdot 10^4$	$81.9 \cdot 10^4$	$33.3 \cdot 10^4$	$20.2 \cdot 10^4$	$15.3 \cdot 10^4$	$11.0 \cdot 10^4$	$7.8 \cdot 10^4$
Allantoin	5.24	$370.1 \cdot 10^4$	$347.5 \cdot 10^4$	$270.0 \cdot 10^4$	$197.2 \cdot 10^4$	$130.9 \cdot 10^4$	$82.7 \cdot 10^4$	$53.1 \cdot 10^4$
Peak 2	5.46	$800.4 \cdot 10^4$	$732.0 \cdot 10^4$	$424.6 \cdot 10^4$	$198.2 \cdot 10^4$	$90.8 \cdot 10^4$	$42.0 \cdot 10^4$	$19.8 \cdot 10^4$

[a] The retention time.

Table 3
Linear regression lines standards of purine metabolites (PMs), the coefficients correlation (r), the standard error in slope (SES) and the standard error in the intercept (SEI)

Compound	Range of PM concentration (μM) in standards	Detection wavelength (nm)	Equation[a]	Correlation coefficient		SES	SEI
				($r_{standard}$)	(r_{urine})[b]		
Allantoin	172–1725	205	$y=4.241\cdot10^{-4}S_n-5$	1.000	0.999	$3.82\cdot10^{-6}$	8
		225	$y=2.040\cdot10^{-3}S_n-9$	1.000	1.000	$2.46\cdot10^{-5}$	10
Uric acid	151–1515	205	$y=3.243\cdot10^{-4}S_n-79$	0.992	0.982	$4.50\cdot10^{-5}$	84
		284	$y=3.104\cdot10^{-4}S_n-1$	1.000	0.994	$3.26\cdot10^{-6}$	8
Hypoxanthine	117–1174	205	$y=9.358\cdot10^{-5}S_n+1.2$	0.995	0.995	$3.02\cdot10^{-7}$	1.6
		250	$y=1.401\cdot10^{-4}S_n+3.6$	1.000	1.000	$4.04\cdot10^{-7}$	1.7
Xanthine	69–696	205	$y=3.316\cdot10^{-4}S_n-14$	0.996	0.966	$1.77\cdot10^{-5}$	19
		267	$y=5.410\cdot10^{-4}S_n-14$	0.998	0.994	$2.22\cdot10^{-5}$	15

[a] S_n and y are the peak areas and purine metabolites concentrations (μM) in a sample, respectively.
[b] The correlation coefficients of purine metabolites assay in urine samples (the range of concentrations – allantoin: 68–1504 μM; uric acid: 25–601 μM; hypoxanthine: 16–414 μM; xanthine: 17–312 μM).

showed no important differences between purine metabolites concentrations in fresh (2 h), non-preserved (25 h) and preserved urine samples. Our experiments showed that the allantoin content seriously declined in a non-preserved urine sample stored for 25 h, while the small xanthine level increased, probably apparently, as a result of background fluctuations. To avoid blocking a guard column, urine samples, especially non-preserved, required filtration through a Millipore 0.2-μm filter (Cole Parlmers).

Considering the above results, HCl and H_2SO_4 can be recommended as preservation agents. Urine should be collected from animals into an appropriate amount of diluted HCl or H_2SO_4 to lower the final pH to below 3 and the final dilution of 1:3–1:6.

Table 4
The comparison between purine metabolites concentration in a fresh (2 h) non-preserved urine (sheep A), non-preserved urine stored for 25 h (sheep A), and preserved urine samples with different agents (sheep A and B)[a]

Preservation agent	Time[b] (h)	Sheep	Allantoin (mg l^{-1})	Uric acid (mg l^{-1})	Hypoxanthine (mg l^{-1})	Xanthine (mg l^{-1})
Without agent	2	A	632	117	52	9.7
(control)	25	A	570	111	51	15.6
HCl	25	A	645	120	53	9.6
		B	526	101	54	73
H_2SO_4	25	A	650	115	52	11.6
		B	549	103	56	74
$CHCl_3$	25	A	604	113	52	12.4
	25	B	521	100	54	71
	27	B	524	100	63	57
	32	B	520	102	62	59
	38	B	521	94	60	57

[a] The ambient temperature was 19–21°C.
[b] The time of urine storage after thawing.

Alternatively, acidified collected urine samples should be vigorously mixed and diluted by a factor of no less than 6 prior to HPLC analysis.

3.1. Reliability of HPLC method

Reproducibility and reliability of the current method was assessed by performing replicate injections of diluted urine samples. As can be seen from coefficients of variation (CVs, 0.29–0.73%) summarized in Table 5, UV monitoring at the longer wavelengths offers better precision of purine metabolites assays as compared with UV detection at 205 nm. The current HPLC method based on the longer wavelength UV detection is suitable for routine analysis of purine metabolites since purine metabolites contents in ovine urine are high in comparison with the obtained LODs and LOQs (Table 5). The low values of the LODs and LOQs point to satisfactory sensitivity of the method, however, another HPLC method [16] based on direct measurements at short UV detection (at 205 nm) offers lower LOD values (0.06 nmol for allantoin, hypoxanthine and xanthine, and 0.1 nmol for uric acid). On the other hand, the precision of that method [16] is worse (CV 3.21–6.64) in comparison with our proposed detection modes (at the short- and long-wavelength UV ranges).

Recoveries of purine metabolites standards from urine samples are presented in Table 6. Accuracy of the HPLC separations was assessed using detection at various UV wavelengths. It is evident from the results obtained using the proposed gradient program that the accuracy of the method is better when the purine metabolites detection was based on UV monitoring at the longer wavelengths. However, both detection methods gave good accuracy of purine metabolites assays; in fact, the obtained recoveries of the added purine metabolites standards were satisfactory for detection at 205 nm and at the longer UV range (i.e., 97.6–114.5% and 97.6–106.8%, respectively). The accuracy of the method was further assessed by determining relationships between the monitoring wavelength (λ_m) and the ratios (R_{urine}) of individual purine metabolite peaks in a calibration standard and urine sample (Table 7). Moreover, these results were compared with relationships between λ_m and ratios (R_{stand}) of purine metabolites in a standard to purine metabolites peaks in another standard (i.e., ''spiked standard''). Comparison of results (Table 7) indicated that peaks corresponding to purine metabolites in urine samples were assumed to be pure in the applied UV ranges, i.e., devoid of interference due to co-eluting peaks of species absorbing in the UV ranges used. On the other hand, beyond the examined UV ranges of purine metabolites monitoring, R_{urine} ratios increased steadily and slightly (as the result of interference) or, rarely, decreased steadily and slightly (as the result of purine metabolites UV signals decreasing and a rise in background fluctuations). Obviously, R_{stand} ratios were invariable also beyond the UV ranges of purine metabolites monitoring in urine samples.

Table 5
Coefficients of variation (CVs), the limits of detection (LODs) and the limits of quantification (LOQs) derived from determinations of purine metabolites in urine samples

Purine metabolite		CV[a] (%)	LOD (nmol)	LOQ (nmol)
Allantoin	205 nm[b]	0.97±0.02	–	–
	225 nm	0.61±0.08	0.16	0.52
Uric acid	205 nm	0.68±0.11	–	–
	284 nm	0.47±0.08	0.21	0.71
Hypoxanthine	205 nm	0.51±0.11	–	–
	250 nm	0.29±0.09	0.23	0.76
Xanthine	205 nm	0.97±0.12	–	–
	267 nm	0.73±0.09	0.70	2.32

[a] The CV values based on four urine samples repeated 3–4 times (dilution and injections).
[b] The detection wavelengths.

4. Conclusion

The current method allows a precise, accurate and specific determination of allantoin, uric acid, hypoxanthine and xanthine in ovine urine. Since urine samples are simply diluted with water (1:6, v/v), the proposed method is devoid of problems associated with pre- and post-column derivatization and with using an internal standard. The current method based on widely available C_{18} columns and PAD of column effluent is suitable for routine analysis of ovine urine. Furthermore, this method can be used to

Table 6
Recoveries (R, %, mean±SD)[a] of purine metabolites standards added to urine samples

Component		Allantoin	Uric acid	Hypoxanthine	Xanthine
Added (μM)		345	303	235	139
	205 nm	104.9±14.2	111.4±5.2	102.0±3.3	107.0±3.4
Recovery (5)[b]					
	$\lambda_{changed}$[c]	99.3±4.3	102.3±3.0	100.2±3.3	102.9±1.5
Added (μM)		230	202	156	93
	205 nm	100.5±5.1	105.8±4.0	102.2±3.6	114.5±7.6
Recovery (6)					
	$\lambda_{changed}$	98.4±1.7	98.9±3.1	99.3±1.1	104.1±1.8
Added (μM)		172	152	117	70
	205 nm	108.6±1.7	111.8±5.7	105.1±1.6	107.4±3.9
Recovery (4)					
	$\lambda_{changed}$	104.8±2.4	105.6±1.3	99.8±1.5	103.7±0.8
Added (μM)		138	121	94	56
	205 nm	97.6±1.4	111.2±1.2	99.5±0.5	107.8±4.9
Recovery (2)					
	$\lambda_{changed}$	97.6±1.3	106.8±0.6	99.7±0.7	106.4±4.3
Added (μM)		69	61	47	28
	205 nm	99.6±7.2	103.0±4.5	102.7±4.7	103.0±7.9
Recovery (8)					
	$\lambda_{changed}$	100.9±2.6	102.6±1.4	100.6±2.3	101.6±5.3
Pooled data:					
	205 nm	102.2±8.3	107.4±5.7	102.7±3.7	107.1±8.2
Recovery (25)					
	$\lambda_{changed}$	100.2±3.3	102.4±3.2	101.1±1.9	102.9±3.9

[a] Recovery was calculated as: R (%)=$(S_1-S_0)\cdot 100\%/S$, where S_0 and S_1 are measurements before and after addition standard of purine metabolites and S is the amount of added purine metabolites.
[b] Number of replicates.
[c] The wavelength of monitoring: allantoin – 225 nm; uric acid – 284 nm; hypoxanthine – 250 nm; xanthine – 267 nm.

Table 7
Relationships between the wavelength (λ_m) of purine metabolites monitoring and ratios of purine metabolites peaks in a standard to purine metabolites peaks in urine sample (R_{urine}) or to purine metabolites peaks in another standard (R_{stand})

Purine metabolite	UV range of monitoring (nm)[a]	RSD (%)[b] of		Relative interval (%)[c] of	
		R_{urine}	R_{stand}	R_{urine}	R_{stand}
Allantoin	203–237	2.04	0.79	5.10	3.02
Uric acid	272–292	0.37	0.21	1.95	1.18
Hypoxanthine	234–262	0.62	0.12	1.89	0.81
Xanthine	247–273	0.64	0.83	1.70	1.91

[a] 2-nm increments.
[b] The relative standard deviation of values R_{urine} or R_{stand}.
[c] The relative interval (%)=$(R_{maximal}-R_{minimal})\cdot 100\%/R_{average}$, where: $R_{maximal}$, $R_{minimal}$ and $R_{average}$ are the maximal, minimal and average of ratio value (i.e., R_{urine} or R_{stand}), respectively.

provide non-invasive estimates of microbial protein supply.

Unfortunately, when the same HPLC procedure was applied to deproteinized plasma of ovine blood, peaks identified as allantoin, uric acid, hypoxanthine and xanthine were not satisfactorily separated from interfering species. Therefore, for a complete, direct analysis of purine metabolites in plasma, the new gradient program should be developed.

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