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Simultaneous measurement of allantoin, uric acid, xanthine and hypoxanthine in blood by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for determining catabolism products of nucleic acids and purines, such as oxypurines (i.e. uric acid, xanthine and hypoxanthine) and allantoin in the blood plasma of ruminants was developed. The plasma was deproteinized with 10% trichloroacetic acid. The method enabled determination of oxypurines without derivatization. Allantoin was determined after conversion with 2,4-dinitrophenylhydrazine to a hydrazone (GLX-DNPH). Separation of converted allantoin, uric acid, xanthine and hypoxanthine derivatives was carried out using two reversed-phase C_{18} columns. The combination of pre-column derivatization and gradient elution with monitoring of the effluent at 205, 254 and 360 nm provides a simple and selective analytical tool for studying oxypurines and allantoin in plasma. The total run time of the HPLC analysis was 60 min. The recovery of the purine derivatives (i.e. oxypurines and allantoin) added to the plasma was between 95 and 106%. Purine derivatives were stable when the processed samples were stored for 7 days at −10°C. The low values of the intra-assay coefficient of variations (2.5–4.6%) and the low values of the detection limits (0.187–0.004 nmol) point to the satisfactory precision and sensitivity of the method.

Keywords: Allantoin; Uric acid; Xanthine; Hypoxanthine

1. Introduction

The amount of bacterial protein formed in the rumen of polygastric animals is an important factor to be taken into account in the modern systems of estimating protein delivery for intestinal digestion. However, determining the amount of bacterial protein leaving the rumen is laborious and involves cannulated animals since the bacterial proteins are mixed with those of feed origin, and the methods used for this purpose are not sufficiently precise [1]. Nucleic acids of bacterial origin are metabolised in the body into purine derivatives (PDs) such as oxypurines (i.e. uric acid, xanthine and hypoxanthine) and allantoin which are excreted in urine. The daily excretion of allantoin and other PDs is correlated with uptake of microbial purines and can therefore provide an index of intestinal flow of the amount of bacterial protein in ruminant animals [2–14].

Various analytical methods for determining PDs in biological samples such as urine or blood plasma have been described [9,15–19]. These procedures are based mainly on the separation by HPLC methods using reversed-phase C_{18}-columns and monitoring at wavelengths ranging from 200 to 218 nm or at 254 nm for PDs other than allantoin. The main advantage of these methods is that urine samples are analysed

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directly, while the plasma samples only need acid deproteinization. Unfortunately, UV detection at short wavelengths (i.e. up to 218 nm) is not selective enough. Because allantoin is poorly retained on a C_{18} column and has a very low molar absorptivity at wavelengths above 220 nm, we converted allantoin to a derivative containing a chromophore group. In order to avoid these problems, we developed an HPLC method in which uric acid, xanthine and hypoxanthine were determined directly, while allantoin was determined following a pre-column derivatization procedure (the Rimini–Schryver reaction) [16,20]. Some authors [21,22] removed uric acid prior to the derivatization of allantoin; however these methods are time consuming and labor intensive. Therefore, we now report a simple method for urine or plasma allantoin analysis in the presence of uric acids, xanthine and hypoxanthine. The combination of the direct determination and pre-column derivatization and separation by HPLC can provide an alternative simultaneous method for determining PDs in the blood or urine of ruminants (in urine directly, in plasma samples after deproteinization).

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany) while allantoin, uric acid, hypoxanthine, xanthine and 2,4-dinitrophenylhydrazine (DNPH) were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical reagent grade and purchased from POCH (Gliwice, Poland). Water was distilled and then deionized prior to use. HPLC-grade water was prepared using a Milli-Q system (Millipore, Toronto, Canada). The mobile phase (solvents A and B) was filtered through a 0.2-μm membrane filter (Millipore). Solvents A and B were degassed by 5 min in vacuum with ultrasonication prior to use.

Thymol blue (a pH indicator) was dissolved in deionized water (0.04% w/v) and then filtered. The derivatizing solution was prepared by dissolving 100 mg of DNPH in 100 ml of 2 *M* HCl and then passing through a membrane filter [16].

2.2. HPLC configuration

A Waters 625 LC system that included a controller for gradient elution and two pumps (Waters Model 501) was used. The apparatus consisted of an absorbance detector Waters Model 486, Waters 712 WISP autosampler, and computer data handling system (all equipment from Waters, Millipore, MA, USA). Development of the analytical method, collection and data integration were performed by using MILLENNIUM 2001 software and a pentium 5P60 computer.

Purine derivatives were separated by means of conventional column chromatography using two serially connected Nova-Pak C_{18}-columns (250 mm×4.6 mm I.D., 4 μm, Waters) connected in series. A 10×6 mm I.D. guard column (Waters) containing reversed-phase C_{18} (30–40 μm) pellicular packing material of the same type was used.

2.3. Analytical solvents and gradient composition

A binary gradient program was used for analysis of PDs in plasma or urine samples (Waters curvilinear program). The following elution solvents were used: solvent A, 0.0025 *M* $NH_4H_2PO_4$ buffered to pH 3.5 with 10% phosphoric acid; solvent B, solvent A–acetonitrile (80:20, v/v). The detector was operated at 205, 254 and 360 nm with an attenuation of 0.050 a.u.f.s. (absorbance unit full scale). The gradient profile is shown in Table 1. The initial flow-rate was 0.9 ml/min, then 1.0 ml/min for 17.1 to 21 min and 1.2 ml/min for 21 to 60 min. When solvent B and the total flow-rate increased, the system pressure rose from 22.8 to 34.1 MPa. Injection

Table 1
Gradient composition

Time (min)	Composition (%)	
	Solvent A	Solvent B
0	100	0
8.3	0.8	99.2 (linearly increased from 7.7 min)
17.0	0.4	99.6 (linearly increased from 8.3 min)
48.5[a]	100	0 (linearly decreased from 47.5 min)

[a] After 48.5 min, the columns was reequilibrated for 11.5 min in 100% solution A (flow-rate=1.2 ml/min).

volumes were 5, 10 or 20 μl. All separations were performed at ambient temperature (19–20°C).

2.4. Analysis of alkaline degradation of uric acid

To a freshly prepared 0.5-ml volume of aqueous $1.023 \cdot 10^{-3}$ *M* uric acid was added 5, 10, 50, 100 or 200 μl of 0.6 *M* NaOH. Tubes were capped and heated at 85°C for 1 h to accomplish degradation of uric acid and the formation of allantoin, and then its hydrolysis to allantoate. Subsequently the mixtures were injected onto the HPLC system and analysed according to the HPLC method described in Ref. [19]), or used for the derivatization with 200 μl of derivatizing solution (DNPH) for 20 min at 85°C. The filtered solutions were used for HPLC analysis.

2.5. Blood and urine sample preparation

Blood samples from the jugular vein of sheep were collected into heparinized tubes (kept in an ice bath) and centrifuged at 1500 *g* for 20 min (at 0–4°C). The plasma was stored at −20°C. On the day of analysis, 3 ml of plasma (0–4°C) were deproteinized with 3 ml of 10% cooled solution (2–4°C) of trichloroacetic acid and centrifuged at ~4000 *g* for 15 min (at 4°C). The supernatant was used for the derivatization procedure.

Urine sheep samples were acidified to a pH below 3 with 1 *M* H_2S0_4. For analysis, urine samples were mixed and then diluted 1:20 or 1:40 with water prior to storage at −15°C.

2.6. Derivatization procedure

A 500-μl sample of plasma or solution of DP standards was pipetted into a small narrow-necked vessel containing 50 μl of the pH indicator. The colour of the reaction mixture was orange if the solution was acidic (pH~1.3). Then, 50 μl of 0.6 *M* NaOH solution were added. If the colour of the solution failed to change to blue (especially for the plasma), more 0.6 *M* NaOH was added. When the colour of a sample changed to blue (pH>9.2), the reaction solution (pH from 9.3 to 11) was heated at 85°C for 60 min. Next, 200 μl of the DNPH solution were added (the colour of the sample became orange–yellow) and the heating was continued for 20 min. All samples were filtered through a 0.2-μm filter (Cole Parlmers) into an autosampler vial.

2.7. Identification and quantification of the compounds

Peaks were identified on the basis of retention times of DP standards injected separately or by adding processed standard solutions to samples. The retention times are expressed as: mean$\pm y$ min of 60 analyzed samples. The accuracy of the procedure was tested by adding known quantities of PDs to the plasma and calculating the percentage recovery.

3. Results and discussion

The main analytical problem in the present study was obtaining suitable separation of allantoin from the interfering compounds of plasma samples. The second problem was achieving good separation of other PDs without a pre-column derivatization. Allantoin separation in urine samples can be achieved by using long columns [9,19]. However, in the plasma samples, many other compounds, such as allantoin, are poorly retained on C_{18}-columns or have high absorbances. Fig. 1 shows chromatograms of processed samples and the final concentrations of the analytes. Thus, to avoid problems due to overlapping peaks, allantoin was converted, prior to HPLC separation, to a chromophore with an aromatic ring and absorbance in the near UV range. The principle was to convert allantoin to glyoxylic acid (GLX) by sequential hydrolysis under weakly alkaline (pH<11) and acidic conditions [16], and to react the GLX with DNPH to form a derivative (2,4-dinitrophenylhydrazone of glyoxylic acid) containing a chromophore with an aromatic ring absorbing in the near UV range [17]. The original concentration of glyoxylic acid (GLX) in the sample can be simply determined by the described derivatization procedure, using water instead of 0.6 *M* NaOH (i.e. without hydrolysis of original allantoin to GLX allantoate [16]). Chen et al. [16] measured in the urine and plasma of sheep and cattle and found concentrations in the order of 6–10 μ*M*. This is a relatively small amount compared with the allantoin

level in sheep and cattle (50–150 μ*M*) found by other authors [16,21,23].

Compared with other methods [15,17], the derivatization with DNPH has the advantage of increased stability of the reagents and the derivative. Moreover, the reactions are not time-consuming, and can be automated. Thus the combination of allantoin derivatization and chromatographic separation by using two long C_{18}-columns (connected in series) provides a suitable procedure for simultaneous determination of allantoin, hypoxanthine, uric acid and xanthine in blood samples. As can be seen in Fig. 2A to C, the procedure resulted in suitable separation and quantification of PDs. Fig. 2 shows chromatograms of standards, plasma and urine samples, and the final concentrations of the analytes. Satisfactory results were achieved due to the use of the gradient program and detection at 205 nm for up to 9 min, at 254 nm from 9 to 25 min, and at 360 nm after 25 min. The converted allantoin was substantially retained on the C_{18}-columns (retention time 37.6±1.6 min vs. 5.4±0.4 min) and was distinct from other endogenous substances in plasma or urine samples (see Fig. 1A and C and Fig. 2A and C). As is shown in Fig. 2, in the system developed in this study, the *syn*- and *anti*-isomers of the allantoin derivative [16,18] appeared in the chromatograms as a single peak (i.e., sum of the unseparated isomers). Thus the peak area is considerably larger than peaks of separated isomers [16], allowing better accuracy and precision of the method for allantoin assay.

Moreover, the allantoin derivative separated by gradient elution is monitored at a unique wavelength (360 nm); therefore other compounds present in plasma or urine samples will not interfere with allantoin derivative. As illustrated in Fig. 2B, com-

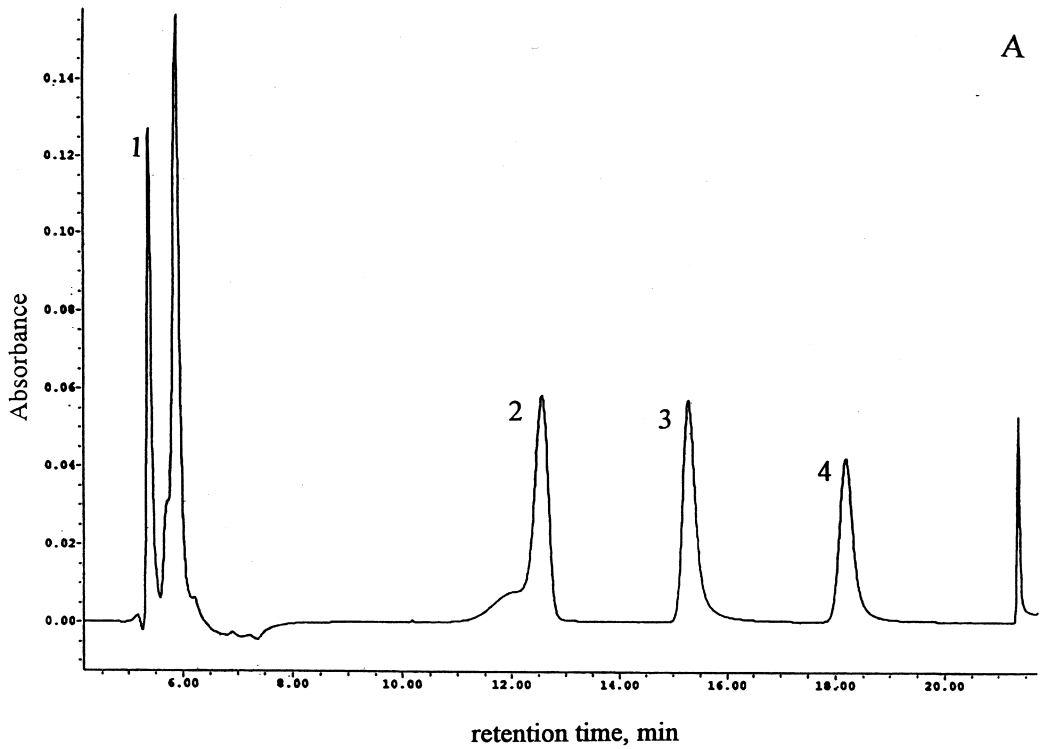


Fig. 1. Chromatograms for (A) standards (165 μ*M* allantoin; 166 μ*M* hypoxanthine, 42 μ*M* uric acid, 66.4 μ*M* xanthine), (B) spiked (with ~47 μ*M* hypoxanthine and ~9.6 μ*M* xanthine) plasma sample (72.8 μ*M* allantoin, 49.0 μ*M* hypoxanthine, 38.5 μ*M* uric acid, 13.4 μ*M* xanthine) and (C) urine sample (133.4 μ*M* allantoin, 7.1 μ*M* hypoxanthine, 35.2 μ*M* uric acid, 24.9 μ*M* xanthine). All samples were analysed directly (without allantoin derivatization). Injection volumes were 10 μl. The detector was set at 205 nm up to 9 min and at 254 nm from 9 min. Peaks: 1=allantoin; 2=hypoxanthine; 3=uric acid; 4=xanthine.

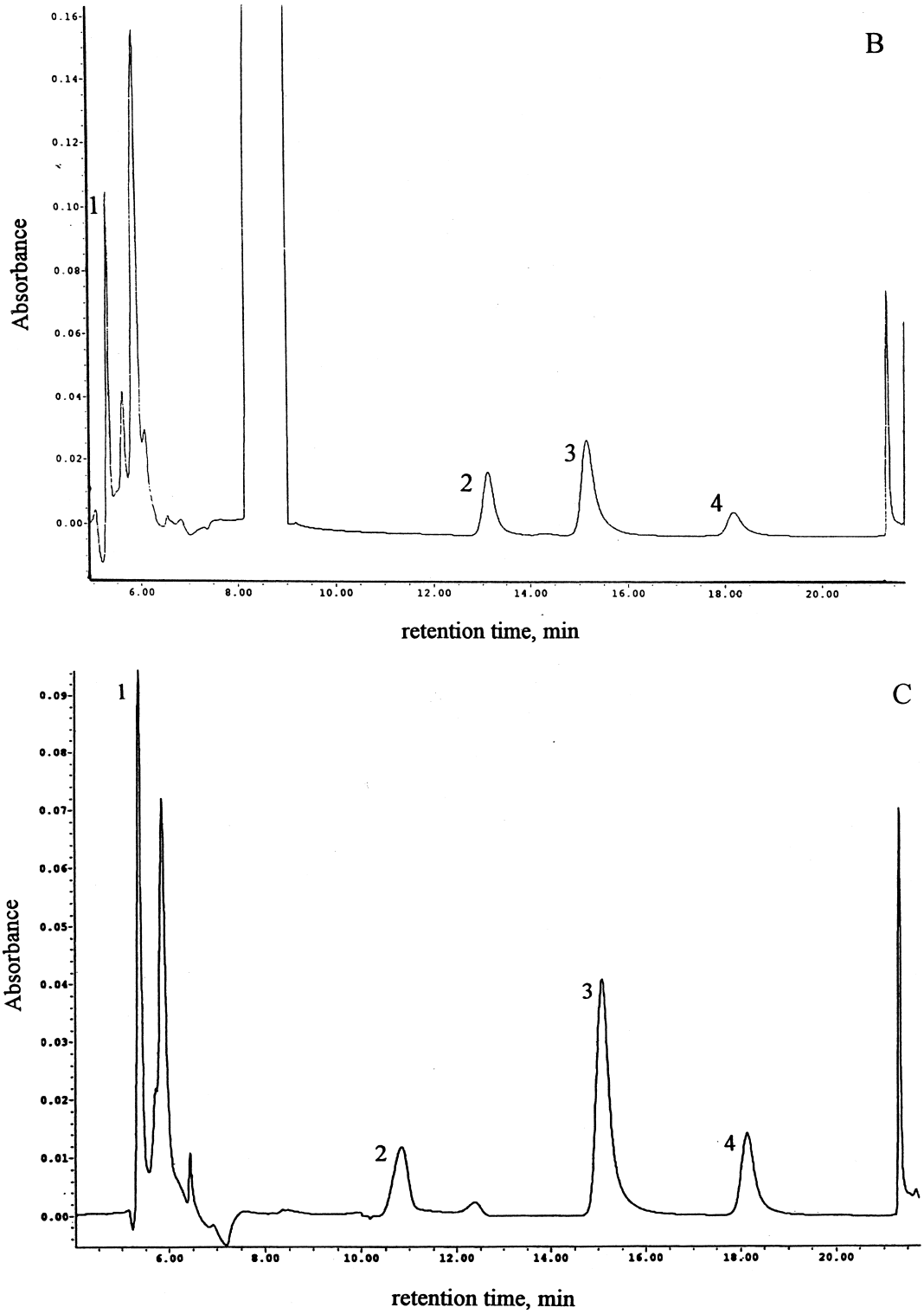


Fig. 1. (*continued*)

plete derivatization of allantoin was achieved because a peak referred to as unchanged allantoin (at 5.4±0.4 min, see Fig. 1B) was absent.

The preliminary experiment showed that a linear relationship between peak response (i.e. formation of derivatized allantoin) and concentration of allantoin was observed up to $0.98 \cdot 10^{-3}$ *M* allantoin concentration in samples (correlation coefficient, r= 0.99898). Above this concentration, the yield of derivatized allantoin formation was less than 100%.

In our procedure the hypoxanthine peak was eluted at 13.3±1.0 min, uric acid at 14.8±1.4 min, xanthine at 18.1±1.6 min (all compounds were monitored at 254 nm) while allantoin had a retention time of 37.6±1.6 min (detector was set at 360 nm). As expected, these peaks were absent from the blank.

The relationship between the concentrations and peak areas was linear over a wide range of concentrations of PD standards. The corresponding equations and correlation coefficients are given in Table 2.

Exposure of uric acid to strong alkali causes its degradation [21,22]. Previous works [21,22] have shown that uric acid is degraded to allantoin under the strongly alkaline conditions (in ~0.1 *M* NaOH at 100°C). Studies were carried out to ensure that the presence of uric acid in the processed samples would not affect the allantoin assay. Table 3 summarizes some of the results obtained. The yield of allantoin formation in weakly alkaline solution (test No. 2–5) is very low (~1%). This is a relatively small amount when compared with the original allantoin levels in the urine or plasma of sheep and cattle. (The uric acid content is lower than the allantoin concentration [9,13,16,18]). Moreover, preliminary studies [22] showed that the amount of allantoin formation

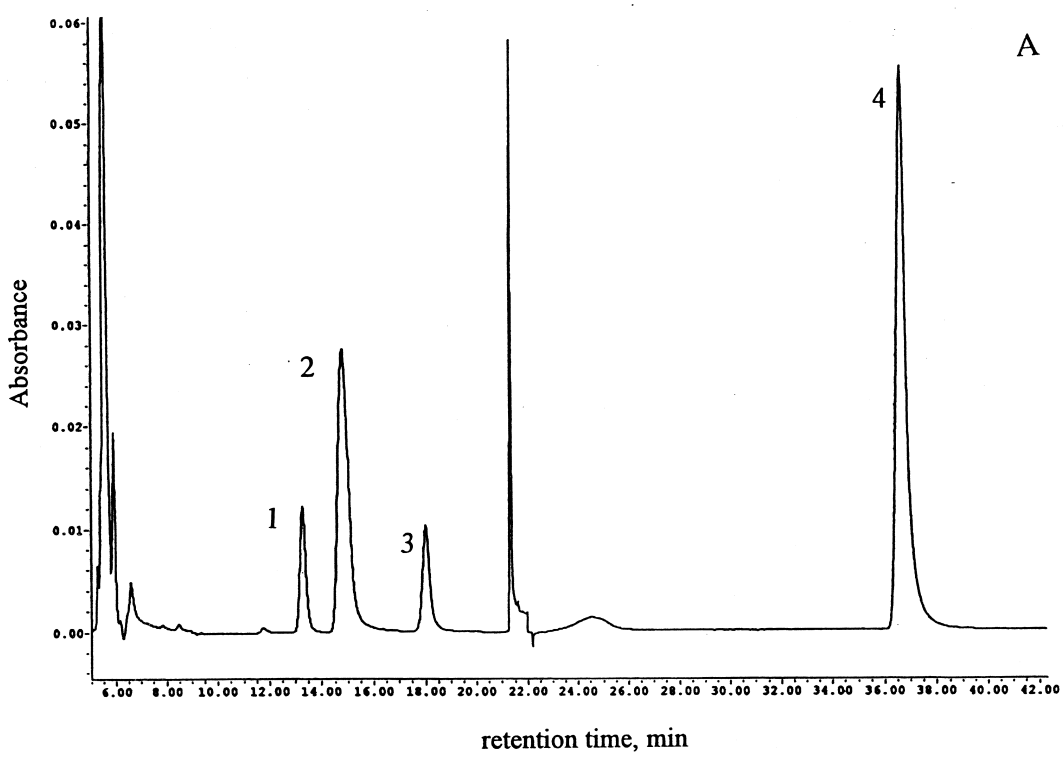
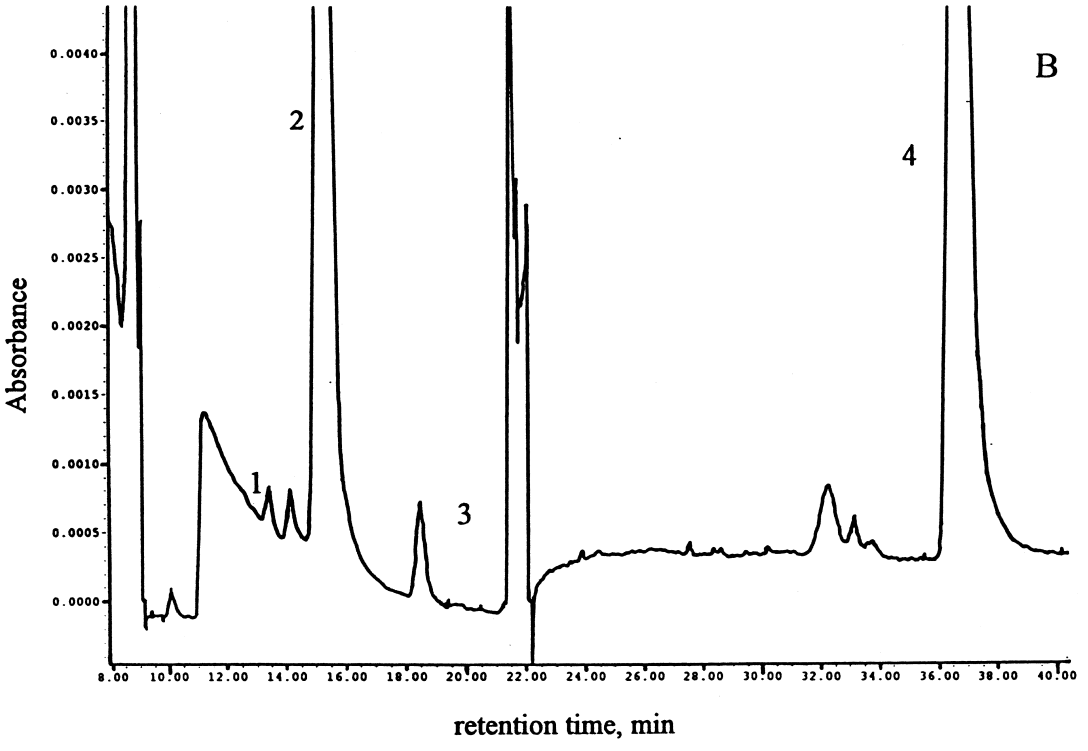


Fig. 2. Chromatograms for (A) standards (37.9 μ*M* hypoxanthine, 47.0 μ*M* uric acid, 25.8 μ*M* xanthine, 62.2 μ*M* allantoin), (B) plasma sample (0.85 μ*M* hypoxanthine, 30.8 μ*M* uric acid, 1.8 μ*M* xanthine, 35.9 μ*M* allantoin) and (C) urine sample (15.7 μ*M* hypoxanthine, 13.9 μ*M* uric acid, 10.3 μ*M* xanthine, 52.6 μ*M* allantoin). All samples were analysed after the derivatization procedure. Injection volumes were 10 μl. The detector was set at 205 nm up to 9 min, at 254 nm from 9 to 25 min and at 360 nm after 25 min of the chromatographic run. Peaks: 1=hypoxanthine; 2=uric acid; 3=xanthine; 4=allantoin derivative.



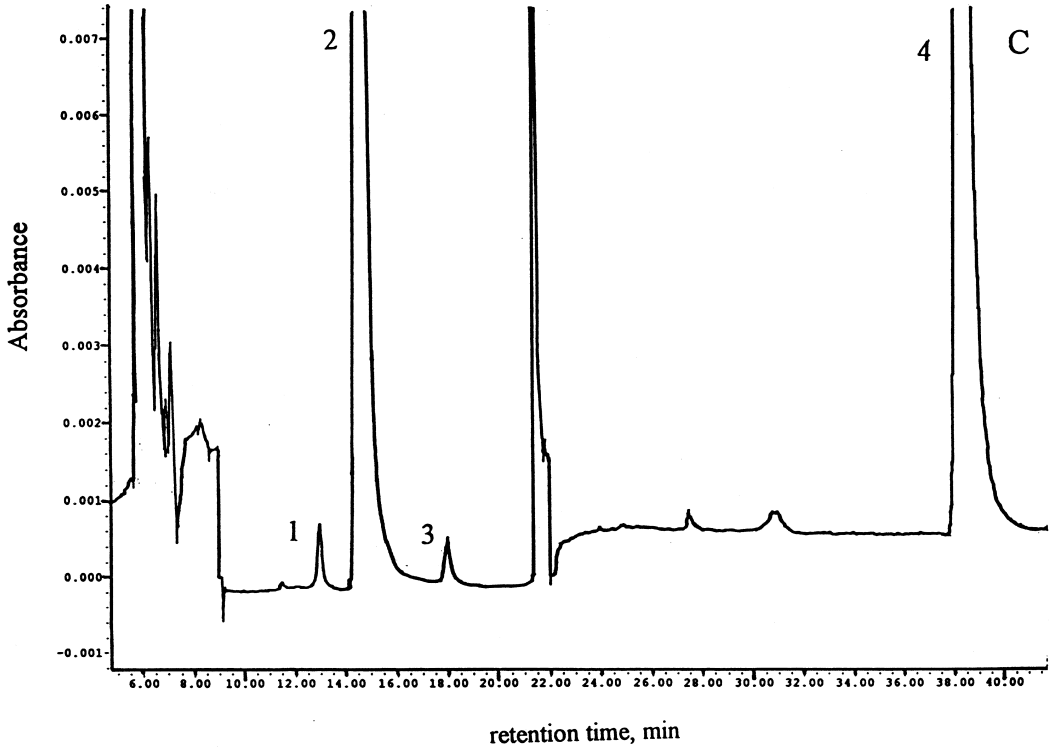


Fig. 2. (*continued*)

Table 2
Linear regression lines of PD standards and coefficients correlation (r)

Compound	Range of concentration (μM)	Equation[a]	Correlation coefficient (r)	Standard error in slope
Allantoin	8.0–234.1	$y=4.259\cdot10^{-5}S_N+0.2$	0.9990	$1.1\cdot10^{-6}$
Uric acid	5.9–421.0	$y=5.611\cdot10^{-5}S_N+0.1$	0.9992	$9.30\cdot10^{-7}$
Hypoxanthine	4.7–220.4	$y=1.965\cdot10^{-4}S_N+0.3$	0.9995	$3.60\cdot10^{-6}$
Xanthine	3.2–468.4	$y=1.112\cdot10^{-4}S_N+1.4$	0.9991	$2.7\cdot10^{-6}$

[a] S_N and y are the peak areas and PD concentrations (μM) in a sample, respectively.

decreased in proportion to the decrease of uric acid concentration.

Alkaline degradation of uric acid may cause an overestimation of allantoin levels in the presence of relatively high concentrations of uric acid and low original allantoin levels in processed samples (e.g. in human body fluids [21,22]. The amount of allantoin produced during degradation of uric acid may be simply determined using a set of processed uric acid standards. Considering the above facts, it is essential to apply a weakly alkaline conditions (pH from 9.3 to 11) to allantoin hydrolysis.

During the method development, studies were also carried out to ensure that the amount of NaOH in the reaction mixture would not restrict the yield of allantoin derivatization. Results showed no differences in the peak response (i.e. GLX-DNPH formation) under weakly alkaline conditions (pH from 9.3 to 11.7). On the other hand, under strongly alkaline conditions ($\geq$0.1 M NaOH) the yield of GLX-DNPH decreased, while, under the chromatographic conditions described in Ref. [19], the formation of an unknown product (retention time: 9.9$\pm$0.5 min) was observed. This product was observed both in allantoin solutions (10^{-3} M) and uric acid solutions (10^{-3} M) after heating at 85°C for 60 min. The yield of the unknown product formation rose with increasing pH value.

The accuracy of the procedure was assessed by examining the recovery of known quantities of the PD standards added to plasma. The results are summarised in Table 4. The recovery estimation was carried out by addition of PD standards to plasma where the pH was adjusted to $>$9.2 with 0.6 M NaOH. When PD standards were added to a highly acidic plasma solution (i.e. before alkalination of the reaction mixture) the recovery of purine metabolites was poor and the accuracy and precision of the method were unsatisfactory. Thus, all plasma samples were spiked after alkalizing the solutions and then the reaction mixtures were carried through the described derivatization procedure. The results of the recovery studies (Table 4) showed that PDs in sheep plasma can be measured reliably using the proposed method, because the obtained recoveries of the added PD standards are satisfactory (95–106%). No

Table 3
Alkaline degradation of uric acid ($1.023\cdot10^{-3}$ M) and formation of allantoin

No. test	Amount of 0.6 M NaOH added to 0.5 ml of uric acid (μl)	Measured converted allantoin concentration (μM)	Uric acid degradation to allantoin (%)
1	0	–	–
2	5	10.2	1.00
3	10	8.8	0.86
4	50	8.1	0.80
5	100	7.7	0.75
6	200	13.8	1.35

Samples were heated at 85°C for 60 min, next, 200 μl of DNPH solution was added and heated at 85°C for 20 min.

Table 4
Recoveries (R %, mean±S.D.)[a] of PD standards added to the plasma and concentrations of added purine metabolites

Derivative of		Hypoxanthine	Uric acid	Xanthine	Allantoin
Added (μM)		30.4	37.8	20.7	51.6
Recovery (%)	A	102.5±2.8 (3)[b]	97.0±1.2 (3)	106.0±1.5 (3)	102.1±3.3 (3)
	B	101.1±2.6 (3)	97.2±1.4 (3)	97.4±0.9 (3)	96.7±1.2 (3)
Added (μM)		19.0	23.6	12.9	32.2
Recovery (%)	A	97.0±3.3 (3)	97.3±2.5 (4)	100.9±3.2 (3)	95.7±2.8 (4)
	B	96.4±0.8 (3)	102.7±4.6 (3)	95.4±4.1 (3)	96.0±4.7 (4)
Added (μM)		7.6	9.4	5.1	12.8
Recovery (%)	A	102.6±1.5 (4)	101.7±1.7 (4)	96.3±1.2 (4)	102.1±3.4 (5)
	B	103.7±1.2 (3)	96.1±1.2 (3)	96.1±1.4 (3)	96.1±3.1 (3)
Pooled data					
Recovery (%)	A	101.1±2.3	99.0±2.0	100.2±1.8	97.1±3.2
	B	100.8±1.8	98.3±3.1	96.8±3.1	96.3±2.9

[a] Recovery was calculated as: $R(\%) = \frac{(S_1 - S_0) \times 100\%}{S}$, where S_0 and S_1 are the measurements before and after addition of PD standards and S is the amount of added PD.
[b] Number of replicates.
A, samples analyzed fresh; B, samples stored at −10°C for 7 days.

changes in recoveries of added compounds were observed when the derivatized plasma solutions were stored for 7 days at −10°C (Table 4).

The intra-assay coefficients of variations (C.V., %, 2.5–4.6%) were calculated by processing aliquots of spiked plasma samples (Table 5), while the second part of Table 5 summarises the detection limits [9] (L_D, nmol) at a signal-to-noise ratio of 3 in plasma. The low values of the intra-assay C.V. and the low L_D values demonstrate the satisfactory precision and sensitivity of the proposed procedure.

When the same procedure was applied to sheep urine samples, peaks identified as hypoxanthine, uric acid, xanthine and allantoin derivatives were satisfactorily separated. Thus, the results from previous [16,19] and the present study suggest that this procedure can be used for determination of PDs in urine samples. The results appear to be reasonable, but recoveries were not measured. Before derivatization, the urine samples should be diluted so that the expected allantoin concentration was between 30 and 320 μM [16,19].

Table 5
Intra-assay C.V. (%) derived from the measurements of PD in plasma samples and the lowest concentration detection limits (L_D) at a signal-to-noise ratio of 3 in plasma

Derivative of	C.V.[a]	C.V.[b]	L_D (nmol)
Hypoxanthine	4.6	3.2	0.187
Uric acid	3.9	2.8	0.004
Xanthine	2.9	2.5	0.007
Allantoin	3.8	3.3	0.004

[a] The intra-assay C.V. values ([16]) based on three samples repeated three or four times (processing and injection).
[b] The intra-assay C.V. values for repeated injections [16] based on three samples each with three injections.

4. Conclusions

The present HPLC method is highly precise for allantoin. Uric acid, hypoxanthine and xanthine can be also simultaneously determined in plasma samples after acid deproteinization. Prior to gradient elution, allantoin is converted to a hydrazone with DNPH. The allantoin derivative and other purine metabolites were separated using two long C_{18} columns and monitoring the effluent at 205, 254 and 360 nm. The HPLC method proposed here provides a relatively sensitive, accurate and simple analytical tool for the estimation of ruminal microbial protein supply to sheep. This procedure uses a non-invasive technique, which only requires collection of blood or urine. This approach holds promise as an alternative to other methods which require cannulation of animals.

References

[1] J.W. Czerkawski, J. Sci. Food Agric. 25 (1974) 45.
[2] A.B. McAllan, R.H. Smith, Br. J. Nutr. 29 (1973) 331.
[3] R.W.E. Watts, in: Proceedings 3rd EAAP-Symposium on Protein Metabolism and Nutrition, Braunschweig, May 1980, Eur. Assoc. Animal Production, Publication No. 27, 1980, pp. 103–117.
[4] A. Antoniewicz, Rocz. Nauk. Zoot. Ser. B 23 (1985) 169.
[5] T. Fujihara, E.R. Ørskov, P.J. Reeds, D.J. Kyle, J. Agric. Sci. Camb. 109 (1987) 7.
[6] J.E. Lindberg, C. Jansson, Swedish J. Agric. Res. 19 (1989) 163.
[7] J.E. Lindberg, Anim. Feed Sci. Technol. 35 (1991) 213.
[8] J. Bacells, J.A. Guada, C. Castrillo, J. Gasa, J. Agric. Sci., Camb. 116 (1991) 309.
[9] J. Bacells, J.A. Guada, J.M. Peiro, J. Chromatogr. 575 (1992) 153.
[10] X.B. Chen, F.D.DeB. Hovell, E.R. Ørskov, D.S. Brown, Br. J. Nutr. 63 (1990) 131.
[11] X.B. Chen, J. Mathieson, F.D.DeB. Hovell, P.J. Reeds, J. Sci. Food Agric. 53 (1990) 23.
[12] X.B. Chen, Y.K. Chen, M.F. Franklin, E.R. Ørskov, W.J. Shand, J. Anim. Sci. 70 (1992) 1534.
[13] R. Puchala, G.W. Kulasek, Can. J. Anim. Sci. 72 (1992) 821.
[14] R. Puchala, J.A. Shelford, W. Barej, G.W. Kulasek, H. Pior, N. Makoni, M. Keyserlingk, J. Anim. Physiol. Anim. Nutr. 69 (1993) 186.
[15] E.G. Young, C.F. Conway, J. Biol. Chem. 142 (1942) 839.
[16] X.B. Chen, D.J. Kyle, E.R. Ørskov, J. Chromatogr. 617 (1993) 241.
[17] X.B. Chen, W. Matuszewski, J. Kowalczyk, J. AOAC. Int. 79 (1996) 628.
[18] M. Czauderna, J. Kowalczyk, J. Anim. Feed Sci. 4 (1995) 351.
[19] M. Czauderna, J. Kowalczyk, J. Anim. Feed Sci. 5 (1996) 433.
[20] R. Borchers, Anal. Biochem. 79 (1977) 612.
[21] J. Lagendijk, J.B. Ubbink, W.J.H. Vermaak, J. Chromatogr. Sci. 33 (1995) 186.
[22] M. Grootveld, B. Halliwell, Biochem. J. 243 (1987) 803.
[23] X.B. Chen, G. Grubic, E.R. Ørskov, P. Osuji, Anim. Prod. 55 (1990) 185.