ELSEVIER

Journal of Chromatography B, 706 (1998) 342–346

JOURNAL OF CHROMATOGRAPHY B

Short communication

Determination of allantoin in bovine milk by high-performance liquid chromatography

K.J. Shingfield*, N.W. Offer

Department of Grassland and Ruminant Science, SAC, Auchincruive, Ayr, Scotland KA6 5HW, UK

Received 15 August 1997; received in revised form 30 October 1997; accepted 31 October 1997

Abstract

Determination of allantoin in bovine milk based on high-performance liquid chromatography (HPLC) is described. Following dilution and filtration, milk samples were analysed directly. Separation and quantification of allantoin was achieved using a Spherisorb 5 NH_2 column (250×4.6 mm ID), acetonitrile–water (90:10, v/v) mobile phase at a flow-rate of 2.0 ml min^{-1}, temperature 20°C and monitoring the effluent at 214 nm. Total analysis time was 10 min. Recovery of allantoin added to milk was 97 (±3.7, n=30)%. Lowest detectable concentration was 1 μmol l^{-1}. Within-day and between-day variability were less than 3%. Advantages of improved retention and separation of allantoin, and less complicated sample preparation exist over current methods. © 1998 Elsevier Science B.V.

Keywords: Allantoin

1. Introduction

Recent studies in ruminant species have demonstrated strong relationships between purines entering the duodenum and the excretion of their metabolites, purine derivatives (PD) in the urine [1,2]. Since, dietary nucleic acids are almost entirely degraded in the rumen [3], purines available for absorption are assumed to be microbial in origin. Consequently, urinary PD excretion has been suggested as a non-invasive index of microbial protein supply. Quantifying urinary PD excretion for a dairy cow is complicated by the requirement for a total urine collection, while its assessment from spot urine samples appears to be unreliable [4]. PDs are also secreted in milk, with allantoin being quantitatively the most important [5,6]. Therefore measurements of milk allantoin excretion could potentially form the basis of an on-farm diagnostic of microbial protein supply in lactating dairy cows [5,6].

Measurements of allantoin in bovine milk have been based on colorimetric analysis [6,7], the principle of which is to hydrolyse allantoin to glyoxylic acid, which in the presence of phenylhydrazine leads to the formation of an hydrazone [8]. However, this approach is subject to criticism due to a lack of specificity [5,8]. In recent years, measurements of allantoin in biological fluids have been performed by isocratic [9–11] or gradient HPLC methodologies [12] based exclusively on reversed-phase C_{18} stationary phases. Methods developed specifically for milk [10,13] are often unsatisfactory due to poor

*Corresponding author. Present address: Department of Animal Nutrition, Institute of Animal Production, Agricultural Research Centre of Finland, Jokioinen FIN-31600, Finland.

0378-4347/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII S0378-4347(97)00549-5

retention and separation of allantoin. This paper describes a simple and precise isocratic method based on an amino stationary phase for the quantitative determination of allantoin in bovine milk.

2. Experimental

2.1. Instrumentation

Analysis was performed using an isocratic HPLC pump Model P100, UV detector Model 200, data-jet integrator (Spectra Physics Limited, Herts, UK) and automatic injector Model 232 (Gilson Medical Electronics, Middleton, WI, USA) fitted with a 60 μl loop. Separation was achieved using a 5 μm Spherisorb amino HPLC column (250×4.6 mm ID) preceded by a normal-phase guard column (10×4.6 mm ID; Phase Separations Limited, Clwyd, Wales, UK).

2.2. Reagents

Allantoin standard assayed above 98% was supplied by Phase Separations Limited. HPLC grade water and far UV grade acetonitrile were supplied by Rathburn Chemicals Limited (Walkerburn, Scotland, UK).

2.3. Mobile phase preparation

Mobile phase was prepared by the addition of 100 ml HPLC grade water to 900 ml of acetonitrile. After passing through a 0.2 μm PTFE filter (Anachem, Bedfordshire, UK), the mobile phase was thoroughly degassed under a vigorous stream of helium for 10 min. The flow of helium was reduced to a steady stream during analysis.

2.4. Chromatographic conditions

Chromatography was achieved under isocratic conditions at a flow-rate of 2.0 ml min^{-1}. Eluted mobile phase was monitored at 214 nm. Allantoin eluted at 3.47 min. Mobile phase flow was reversed after 4 min. Column backflushing continued for 4 min to remove longer eluting compounds from the column. The flow of mobile phase was restored for 2 min prior to sample injection. Sample injection volume was 60 μl. Separation of allantoin was achieved at 20°C, with a total run time of 10 min. Peak purity was assessed by comparison of allantoin UV spectra (range 190 to 600 nm, at 4 nm intervals) in standards with milk samples. Column dead time was determined using chloroform.

2.5. Allantoin standard preparation

Stock allantoin standard solution was prepared by dissolving 0.0158 g of allantoin in one litre of 90% (v/v) aqueous CH_3CN resulting in a concentration of 0.1 m*M* and stored at 4°C for one month. Additional 0.05 and 0.025 m*M* standard solutions were prepared daily by dilution of stock standard with 90% (v/v) aqueous CH_3CN. Daily calibration was performed following triplicate injections of all standard solutions. Quantification was achieved by regression analysis of allantoin peak area against concentration.

2.6. Sample preparation

Milk samples were homogenised using a T1500 homogeniser (Ystral, Dottingen, Germany). Five hundred μl aliquots of milk were diluted with 4.5 ml of HPLC grade acetonitrile and vortex-mixed for 15 s. A 2 ml aliquot of this mixture was passed through a 13 mm disposable syringe filter containing a 0.45 μm PTFE membrane (HPLC Technology, Cheshire, UK) into a HPLC vial.

3. Results and discussion

3.1. Chromatographic separation

During the initial stages of method development the effects of mobile phase composition on allantoin retention were evaluated. Increasing the proportion of acetonitrile contained in the mobile phase increased allantoin retention time and capacity factor (Table 1). Mobile phase containing 90:10 acetonitrile–water resulted in the best compromise between allantoin retention and analytical run time. Standard and sample chromatograms are shown in Figs. 1 and 2, respectively.

Peak identification was performed by the addition

Table 1
Influence of mobile phase composition on allantoin retention time (min)

Mobile phase composition acetonitrile–water (v/v)	Allantoin retention time (min)	Allantoin capacity factor (k')[a]
30:70	1.55	0.57
50:50	1.87	0.89
70:30	1.98	1.01
80:20	2.73	1.77
90:10	3.49	2.54
95:5	4.34	3.40

[a] Calculated as: $k'=(t_r-t_o)/t_o$ [14] where: k' is the compound capacity factor; t_o is the column dead time (determined as 0.99 min, assuming that chloroform is not retained on the column); t_r is the compound retention time (min).

of allantoin standard to milk samples. Peak purity was assessed to check for chemical interference. Comparison of allantoin standard UV spectra with peaks identified as allantoin in milk samples indicated close spectral matches (Fig. 3). Consequently, peaks corresponding to allantoin in milk samples were assumed to be pure and devoid of interference due to co-eluting peaks.

Poor retention and separation of allantoin remains the main drawback with existing HPLC techniques [9–12]. Allantoin capacity factors for these methods typically fall short of the ideal 1 to 10 range [14]. Data provided by Hicks [15] indicated that even the most suitable stationary phases for allantoin analysis, resulted in capacity factors below 0.7. In the current work, employing an amino stationary phase to achieve chromatography lead to a substantial increase in capacity factor to 2.54, which could potentially be increased. Increases in allantoin retention time and capacity factor resulted in improved separation compared to existing techniques based on C_{18} stationary phases.

3.2. Accuracy of the method

Two calibration curves (range in concentration of 2.0–8.0 and 10–50 μmol l^{-1}, respectively) were

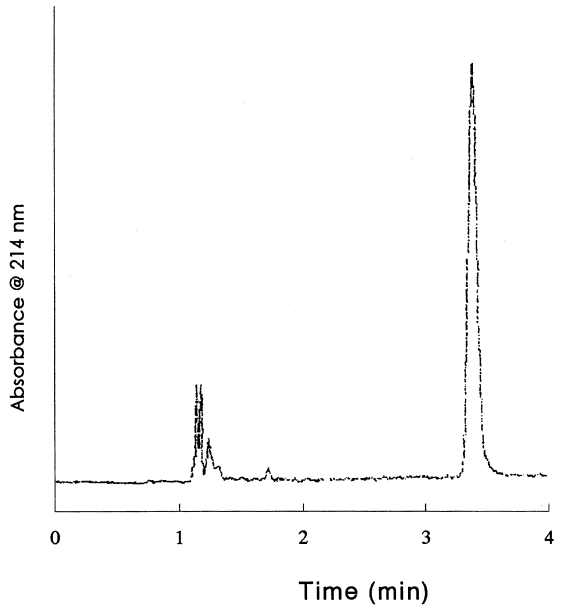

Fig. 1. Chromatogram of allantoin standard.

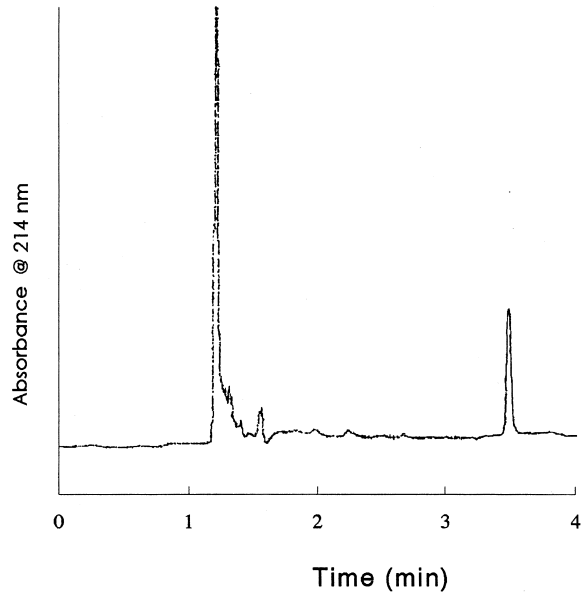

Fig. 2. Chromatogram of milk sample.

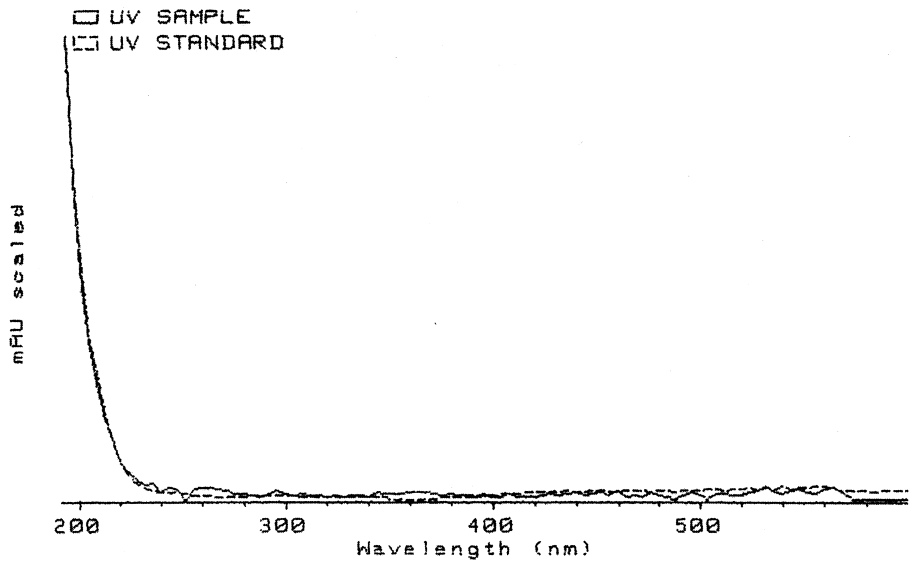

Fig. 3. UV spectra of allantoin in a calibration standard and bovine milk sample.

prepared to evaluate the relationships between peak area and allantoin concentration. Calibration curves were constructed by performing quadruplicate injections at five allantoin additions. Regression equations for standards were calculated as $y=2886$ $(\pm 34.5)x+38$ (± 2.1) and $y=2655$ $(\pm 17.8)x-149$ (± 5.9) over the concentration ranges of 2.0–8.0 and 10–50 μmol l^{-1}, respectively. Applying the standard addition method to milk gave equations of $y=2805$ $(\pm 189.9)x+13271$ (± 2805) and $y=2644$ $(\pm 82.1)x+6767$ (± 597). In all instances, relationships between peak area and added allantoin concentration were linear with regression coefficients above 0.96.

Comparison of standard and sample peak area responses to allantoin addition (regression gradient) indicated recoveries of 97.2 and 99.6% for allantoin additions ranging between 2.0–8.0 and 10–50 μmol l^{-1}, respectively. Accuracy was further assessed by determining the recovery of known quantities (10–60 μmol l^{-1}) of allantoin added to milk (Table 2). Mean recovery was 97 (± 3.7, $n=30$)% which agrees closely with the values obtained by regression.

In complex extraction procedures, it is essential to use an internal standard [12]. Two internal standards allopurinol [12] and phenobarbitone [16] were evaluated. Although peak area responses obtained by standard addition were linear for both compounds, their use was precluded due to co-elution with unidentified compounds present in milk. Since recoveries of allantoin added to milk approached 100%, the inability to use an internal standard was not considered a major disadvantage. Determination of allantoin in milk using existing techniques involves complicated sample defatting and deproteinising procedures prior to analysis. Since allantoin added to milk was quantitatively recovered, the current method allows simple sample preparation prior to analysis.

Reproducibility assessed by performing five replicate injections of five milk samples within a day, for

Table 2
Recovery of allantoin added to milk samples (mean±S.D.)

Allantoin addition (μmol l^{-1})	No of samples tested	Recovery of allantoin added (%)[a]
10	10	96.1 (±1.9)
30	10	97.9 (±4.5)
60	10	97.2 (±4.0)
Pooled data	30	97.2 (±3.7)

[a] Recovery calculated as recovery (%)=$[\{(C_1-C_0)\times 100\}/A]$ where: C_0 and C_1 are determined allantoin concentrations before and after allantoin addition, and A is the quantity of allantoin added.

three consecutive days indicated between-day and within-day coefficients of variation of 1.7 and 2.2%, respectively. Precision of the current method compares well with existing techniques which utilise C_{18} stationary phases [10,13]. Detection limits were determined at a signal-to-noise ratio of 3:1, and corresponded to an allantoin concentration of 1 μmol l^{-1}. In order to preserve the analytical column, guard columns were replaced after 100 sample injections.

Milk allantoin concentrations were found to range between 60–300 μmol l^{-1} depending upon diet and production level, which is in agreement with values of between 159 and 237 μmol l^{-1} reported by Giesecke et al. [5]. Allantoin concentrations in bovine milk are similar to those in plasma (118–185 μmol l^{-1} [5,17]) but are much lower than urinary concentrations which typically range between 0.7 and 29.4 m*M* [4].

A sensitive and precise method has been developed to quantify allantoin in bovine milk with advantages of: (i) simple sample preparation, (ii) prolonged column lifetime due to backflushing and (iii) improved retention and separation of allantoin over existing HPLC techniques.

Acknowledgements

The authors would like to thank Mr. K. McIssac for technical assistance during this work. K.J. Shingfield gratefully acknowledges the Ministry of Agriculture, Fisheries and Food for provision of financial support during this work. SAC receives financial support from the Scottish Office Agriculture and Fisheries Department.

References

[1] X.B. Chen, F.D.De.B. Hovell, E.R. Ørskov, D.S. Brown, Br. J. Nutr. 63 (1990) 131.
[2] J. Verbic, X.B. Chen, N.A. Macleod, E.R. Ørskov, J. Agric. Sci. Camb. 114 (1990) 243.
[3] A.B. McAllan, Proc. Nutr. Soc. 41 (1982) 309.
[4] K.J. Shingfield, N.W. Offer, A.M. Sword, Anim. Sci. 60 (1995) 18A.
[5] D. Giesecke, L. Ehrentreich, M. Stangassinger, F. Ahrens, J. Dairy Sci. 77 (1994) 2376.
[6] P. Susmel, M. Spangero, B. Stefanon, C.R. Mills, Lives. Prod. Sci. 44 (1995) 207.
[7] M. Kirchgessner, M. Kreuzer, J. Anim. Phys. Anim. Nutr. 54 (1985) 141.
[8] E.G. Young, C.F. Conway, J. Biol. Chem. 142 (1942) 839.
[9] W. Tiermeyer, D. Giesecke, Anal. Biochem 123 (1982) 11.
[10] R. Rosskopf, H. Rainer, D. Giesecke, Arch. Anim. Nutr. 41 (1991) 411.
[11] M.T. Diez, M.J. Arin, J.A. Resines, J. Liq. Chromatogr. 15 (1992) 1337.
[12] J. Balcells, J.A. Guada, J.M. Peiro, D.S. Parker, J. Chromatogr. 575 (1992) 153.
[13] W. Tiermeyer, M. Stohrer, D. Giesecke, J. Dairy Sci. 67 (1984) 723.
[14] J.W. Dolan, L.R. Synder, Troubleshooting LC systems, Humana Press, Clifton, New Jersey, 1989.
[15] M. Hicks, L.S. Wong, R.O. Day, Anal. Biochem. 210 (1993) 428.
[16] R. Dennis, C. Dezelak, J. Grime, Acta Pharm. Hungarica 57 (1987) 267.
[17] J. Balcells, D.S. Parker, C.J. Seal, Comp. Biochem. Physiol. 101B (1992) 633.