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# A highly efficient method for determination of allantoin in ruminal digesta, ovine urine and blood serum by HPLC

**M. Czauderna[1], J. Kowalczyk, J. Skomiał and K.M. Niedźwiedzka**

*The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences 05-110 Jabłonna, Poland*

ABSTRACT

A sensitive HPLC method for direct determination of allantoin in ruminal digesta, urine and blood serum of sheep is described. Blood serum and urine samples were diluted 1:3 with deionized water. Separation and quantification of allantoin was achieved using three long Nova Pac $C_{18}$-columns (Waters) and UV detection at 205 and 215 nm. The low coefficient of variation and satisfactory recovery of allantoin added to assayed samples (~100%) provide a suitable tool for routine quantification of allantoin in a large number of urine, ruminal digesta and blood serum samples.

KEY WORDS: allantoin, urine, blood serum, sheep, liquid chromatography

## INTRODUCTION

Urinary purine metabolites (PM; i.e. primarily allantoin or, additionally, oxypurines: hypoxanthine, uric acid and xanthine) have been proposed as markers of microbial protein synthesis. These measurements are, then, an alternative, non-invasive method based on the principle that purine metabolites are derived mainly from the nucleic acids of microorganisms flowing out from the rumen (Gonzalez-Ronquillo et al., 2003; Kowalczyk et al., 2003). Unfortunately, the relationship between the duodenal supply of nucleic acids and the urinary excretion of allantoin or PM is usually obscured by an endogenous fraction coming from the turnover of nucleic acid in tissues and incomplete urinary recovery of purines (Orellana-Boero et al., 2001). Therefore, it is essential to develop a versatile, accurate and very sensitive analytical method for routine determination of allantoin in biological samples derived from ruminants.

[1] Corresponding author: e-mail: m.czauderna@ifzz.pan.pl

## MATERIAL AND METHODS

Allantoin was obtained from Sigma (cat. No. A-7878), while HPLC-grade acetonitrile was purchased from Lab-Scan (cal. No. C02C11X). Other chemicals were of analytical reagent grade and purchased from POCh (Poland). Eluent I was prepared from 2.5 mM $NH_4H_2PO_4$ adjusted to pH 3.5 with phosphoric acid, while eluent II was acetonitrile. Water was obtained from a Milli-Q system (Millipore) and all eluents were filtered through a 0.45 μm membrane filter. The instrument used, a Waters 625LC system, consisted of a controller for gradient elution, two Waters 515 pumps, Waters ™717 plus WISP autosampler, Waters AF in-line degasser and Waters 996 photodiode array detector (DAD). Data acquisition was performed on a Compaq computer using Millennium[32] software (v. 4.0). The DAD was operated in the UV range from 195 to 370 nm with spectral resolution of 1.2 nm and measurement frequency of 1 spectrum per sec. The columns used were two Nova-Pak columns (4 μm, 300 × 3.9 mm, Waters): a shorter Nova Pak column (4 μm, 300 × 3.9 mm, Waters) in conjunction with a guard column. The gradient program (Table 1) was used for fractionation of allantoin from endogenous species present in assayed samples derived from sheep. Ovine urine samples were diluted 1:3 with water. Blood samples were collected in heparinized tubes and immediately centrifuged at 1500 *g* for 20 min at 2-4°C. The obtained blood serum samples were diluted 1:3 with water and then immediately injected onto an HPLC column. Ruminal digesta was filtered and then centrifuged at 1500 *g* for 20 min. Obtained rumen fluids were analysed directly or after *pre*-concentration[2]. The injection volume of assayed samples was 20-40 μl.

TABLE 1

Binary gradient programs used for analysis of allantoin in samples derived from sheep (column temperature of 25°C)

| Time, min | Flow-rate, ml $min^{-1}$ | Eluent I (2.5Mm $NH_4H_2PO_4$), % | Eluent II (acetonitrile), % |
|---|---|---|---|
| 12.3 | 0.70 | 100 | 0 |
| 12.6 | 0.70 | 100 | 0 |
| 14.0 | 0.70 | 5 | 95 |
| 16.0 | 0.80 | 5 | 95 |
| 19.5 | 1.00 | 5 | 95 |
| 22.0 | 1.20 | 5 | 95 |
| 31.7 | 1.20 | 5 | 95 |
| 32.0 | 0.85 | 100 | 0 |
| 37.0 | 0.70 | 100 | 0 |
| 60.0 | 0.70 | 100 | 0 |

[2] 2-3 ml of a ruminal fluid was evaporated to 200-300 μl under a stream of nitrogen, respectively (at a temperature of 40-43°C)

## RESULTS AND DISCCUSION

The key step in the analysis of allantoin is complete separation from numerous endogenous substances present in urine and rumen fluid samples. Unfortunately, in rumen fluid, urine and blood serum samples, allantoin, like many other unidentified species, is poorly retained on reversed-phase columns and has a similar relatively high absorbance only at short wavelengths (<240 nm, i.e. absence of chromophore groups) (Czauderna and Kowalczyk, 2000). Moreover, the UV absorption spectra of allantoin and these other species bear a close resemblance. Thus, to avoid the problems due to overlapping peaks and poor detection specificity, three long columns containing dimethyloctadecylsilyl-bonded amorphous silica were used. Indeed, the combination of direct UV detection (at 205 or 215 nm) and a high-resolution chromatographic system by using long $C_{18}$-columns connected in series provides a very selective tool for routine quantification of allantoin in urine, blood serum or in ruminal digesta of sheep after infusion of allantoin into the rumen (Figure 1). Fortunately, as can be

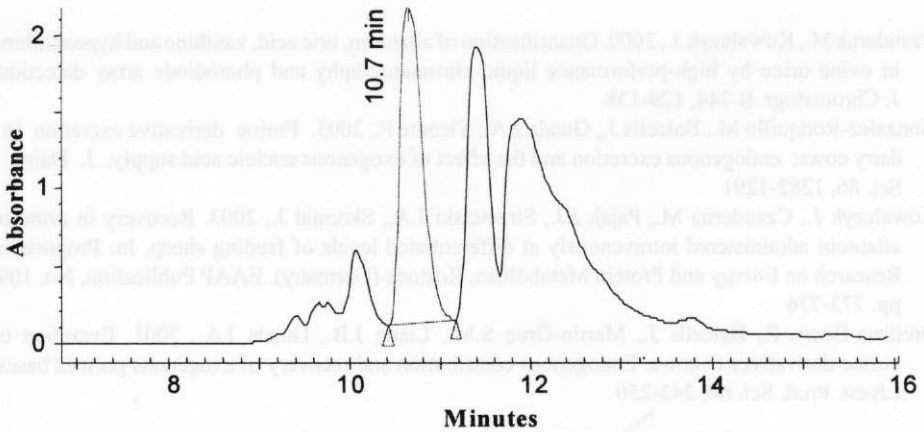


Figure 1. Part of a typical HPLC chromatogram for an ovine urine sample using the current HPLC method and UV detection at 215 nm; the allantoin peak eluted at 10.7± 0.1 min

seen from detailed chromatographic studies of rumen fluid and blood serum samples, satisfactory results were achieved because the assayed samples had less of a complex mixture of endogenous species, especially those eluted after 11 min of a chromatographic run. The low coefficient of variation (1-1.5%), as well as the average recoveries of allantoin added to assayed samples (98-103%) proved that the proposed HPLC method is an adequate tool for routine determination of low levels of allantoin in ovine urine and blood serum samples (i.e. $\sim 3 \cdot 10^{-5}$ M using monitoring at 205 nm). The combination of the *pre*-concentration procedure

and HPLC separation with UV detection at 205 nm enabled the quantification of trace amounts of allantoin (~$5 \cdot 10^{-6}$M) in ruminal digesta after infusion of allantoin into the rumen. The average recovery of the allantoin standard added to ruminal digesta and urine (from 0.1 to 2.0 mM) ranged from 95 to 103%, while the inter-assay C.V. based on 3 samples (processing and 40 µl injection) was less than 5%.

## CONCLUSIONS

The described method using a non-invasive technique enabled simple and rapid analysis of allantoin in ovine urine, blood serum and ruminal digesta. Our procedure avoids problems associated with colorimetric analyses, *pre*-or *post*-column derivatization and deproteinization of blood samples. Our highly sensitive and precise method for determination of allantoin can be suitable for studying the magnitude of the endogenous fraction coming from the turnover of nucleic acids in tissues and the incomplete urinary recovery of infused purines.

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## STRESZCZENIE

**Wysokosprawna procedura oznaczania alantoiny w treści żwacza, moczu i surowicy krwi owiec metodą HPLC**

Opisano czułą i selektywną metodę HPLC oznaczania alantoiny w treści żwacza, moczu i surowicy krwi owiec. Próby rozcieńczono 1:3 wodą dejonizowaną. Prezentowana metoda pozwala na bezpośrednie oznaczanie alantoiny w badanych materiałach, wykorzystując detekcję UV przy długości fali 205 lub 215 nm. Dzięki użyciu trzech długich kolumn $C_{18}$ z odwróconą fazą uzyskano zadowalające oddzielenie alantoiny od innych endogennych związków obecnych w analizowanych próbach. Niski współczynnik zmienności, niemal 100% odzysk dodanej alantoiny do badanych prób dowodzi, że opisana metoda może być wykorzystana w rutynowych badaniach. Opracowano także metodę oznaczania alantoiny po ~10-krotnym zatężeniu płynu żwaczowego.