

Quantitative determination of an extremely polar compound allantoin in human urine by LC-MS/MS based on the separation on a polymeric amino column

Antoine Berthemy, John Newton, Danlin Wu, Deborah Buhrman *

Sanofi Pharmaceutical Research Division, 9 Great Valley Parkway Malvern, PA 19355, USA

Received 25 September 1997; received in revised form 20 August 1998; accepted 9 September 1998

Abstract

Quantitative determination of allantoin in biological matrix poses a challenge to bioanalysis due to the extreme polarity of allantoin. The molecule exhibits virtually no retention on any of the available hydrophobic HPLC packing materials. In this study, an assay was developed for the LC-MS/MS quantitation of allantoin in human urine using a polymeric amino column to achieve the necessary separation. The urine samples were prepared by a single-step solid phase extraction (SPE) procedure. The extracted samples were analyzed on a jordi-gel DVB polyamine column (operated under an acetonitrile/water gradient with water as the stronger mobile phase) interfaced with a Finnigan TSQ 7000 mass spectrometer. Negative electrospray ionization (ESI) was employed as the ionization source. Allantoin and its internal standard ($^{13}\text{C}_1$, $^{15}\text{N}_1$ -allantoin) were detected by use of single reaction monitoring (SRM) mode. The method was validated in the concentration range of 14.6–213 $\mu\text{g ml}^{-1}$, with within and between run accuracy and precision both $< 7\%$. The method has been successfully applied to clinical sample analysis. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Allantoin; LC-MS/MS; Bioanalysis; Human urine; Quantitative determination; Polar compound; Pharmaceuticals

1. Introduction

Allantoin (Fig. 1) excreted in urine can be used as an indicator for the state of purine metabolism in most mammals. Recently it has been suggested that urinary allantoin can serve as an indicator of microbial protein flowing to the small intestine [1]. There have been wide spread interests in

quantitative analysis of urinary allantoin, especially in the areas of food, nutrition, and agriculture. Historically, colorimetric assays based on the Rimini–Schryver reaction [2] were used for

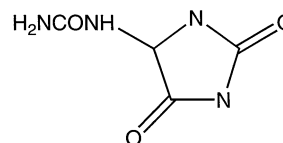


Fig. 1. The structure of allantoin.

* Corresponding author. Tel.: +1-610-8896975; fax: +1-610-8896356.

quantitation. These assays lack specificity often resulting in overestimation due to the presence of interfering material [2], and are sensitive to the experimental conditions employed [3]. With the advancement of HPLC in the late 70's and early 80's, methods for the quantitation of urinary allantoin based on HPLC (mostly reversed phase) have been reported [3–5]. However, due to the extreme polar nature of allantoin, the capacity factors (k') in the published methods were very small on reversed phase HPLC columns, and resolution was difficult to achieve. There have been efforts to employ two columns connected in series (500 mm total length) to maximize the theoretical plate number [3]. The most recent HPLC method and probably the most advanced is by Chen et al. [6], using a pre-column derivatization scheme in which allantoin was sequentially hydrolyzed under alkaline and acidic conditions, the product glyoxylic acid (GLX) was allowed to react with 2,4-dinitrophenylhydrazine to form GLX 2,4-dinitrophenylhydrazone which was detected at 360 nm.

Uric acid level can be dramatically elevated in cancer patients treated with chemotherapy, which may lead to renal toxicity. Urate oxidase decreases the uric acid level by conversion of uric acid to allantoin, which is less toxic due to higher solubility in urine and more readily excreted renally. A urate oxidase developed by Sanofi was evaluated in clinical trials, and a bioanalytical method for the allantoin determination in human urine was needed to support these studies. The existing HPLC methods in the literature (most of them are for animal urine) were found, in our initial methods evaluation, to have inadequate resolution for human urine analysis. Allantoin exhibited little or no retention in the reversed phase columns evaluated, even under 100% aqueous elution. The pre-column derivatization method [6], although specific and sensitive, requires extensive sample preparation, making it difficult to analyze large number of samples routinely. In this report, a simple, specific and efficient LC-MS/MS method was developed for the quantitative determination of allantoin in human urine. The sample preparation was based on a rapid solid phase extraction procedure in which the loading fraction was directly injected onto the LC-MS/MS (i.e. there were

no washing and elution steps involved). The chromatographic separation, based on the strong retention of allantoin, was achieved by use of a polymer based amino column. A shallow gradient of acetonitrile/water was applied with water being the stronger elution solvent. Negative electrospray ionization (ESI) and single reaction monitoring (SRM) were employed in the mass spectrometry detection.

2. Experimental

2.1. Materials

Allantoin was obtained from Sigma (St. Louis, MO). Stable isotope labeled $^{13}\text{C}_1$, $^{15}\text{N}_1$ -allantoin (isotopic purity >99.5%) was from Isotec (Miami, OH). Water and acetonitrile (HPLC grade) were from J.T. Baker (Phillipsburg, NJ). Helium, argon, and nitrogen (all research grade) were from Air Products and Chemicals (Allentown, PA). Solid phase extraction cartridges, SPEC Plus C_{18} AR (3 ml barrel, 15 mg packing), were from Ansys (Irvine, CA). Control human urine was obtained from healthy volunteers in house.

2.2. Sample preparation and LC-MS/MS analysis

Solid phase extraction (SPE) cartridge was conditioned with 1 ml of acetonitrile and 1 ml of water. The water was removed under vacuum. The urine sample (0.2 ml), after fortification with internal standard (0.05 ml, final concentration $50 \mu\text{g ml}^{-1}$), was centrifuged at 3500 rpm for 5 min and the supernatant was loaded to the pre-conditioned SPE cartridge. The loading fraction was collected and injected (25 μl) to the LC-MS/MS. The recovery of allantoin was quantitative (no absorption to SPE).

An HP 1090 HPLC system consisted of an autosampler, two pumps for gradient solvent delivery, a column oven operated at 60°C, and a diverge valve to direct LC effluent to mass spectrometer in the elution window 6–16 min. A $250 \times 4.6 \text{ mm}$, 5 μm , jordi-gel DVB polyamine

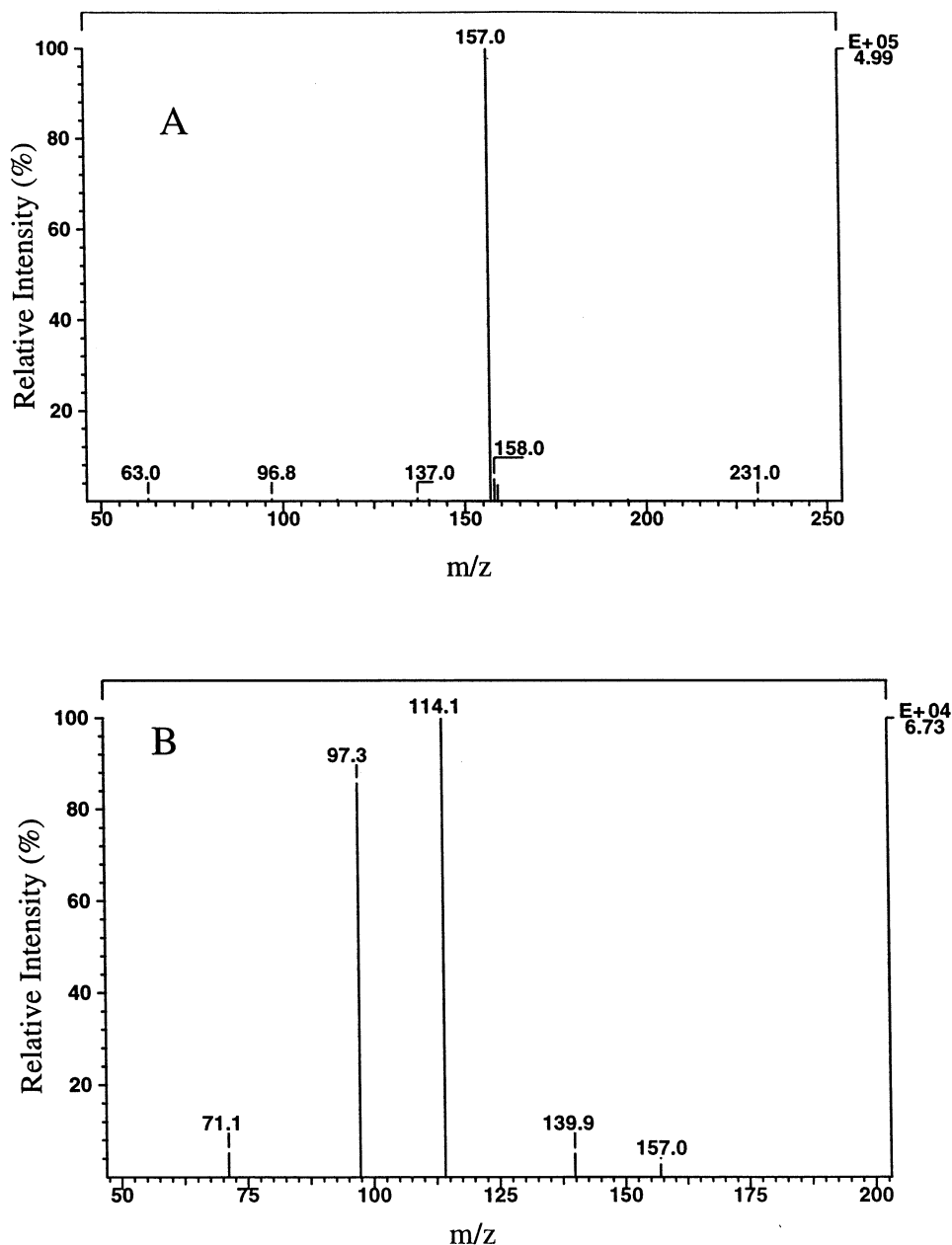


Fig. 2. Full scan (A, m/z 157) and product ion (B, m/z 114) mass spectra of allantoin under negative electrospray ionization.

column was used. The elution was accomplished by a water (A)/acetonitrile (B) gradient with the following profile: $t = 0, 5, 7, 15, 16.5,$ and 19 min, $B\% = 100, 92, 90, 90, 100,$ and 100 , respectively; $t = 0, 5.1, 15, 16.5,$ and 19 min, flow rate = $1.5, 1.0, 1.0, 1.5,$ and 1.5 ml min⁻¹, respectively. The

HPLC effluent was directed through the diverge valve to a Finnigan ESI interface on a Finnigan TSQ 7000 triple quadrupole mass spectrometer. The capillary heater was set at 250°C . The spray voltage was fixed at 4.5 kV. The collision gas (argon) pressure was established at 2.5 mTorr; the

collision energy (voltage in the collision cell) was 16 V. An electron multiplier voltage of 1600 V was used. The sheath and auxiliary gas settings were 100 psi and 30 cc min⁻¹, respectively. The dwell time was 500 ms. The instrument was operated in the negative mode. The parent ion of allantoin [M–H]⁻ at *m/z* 157 (159 for internal standard) was admitted to the first quadrupole (Q1). After the collision induced fragmentation in Q2, the product ion at *m/z* 114 (116 for internal standard) was monitored in Q3. Unit resolution (at half peak height) was used for both Q1 and Q3. Data processing was carried out using a Finnigan QuanGuide data analysis program. Peak area ratios based on SRM of allantoin (*m/z* 157 → 114) and the internal standard (*m/z* 159 →

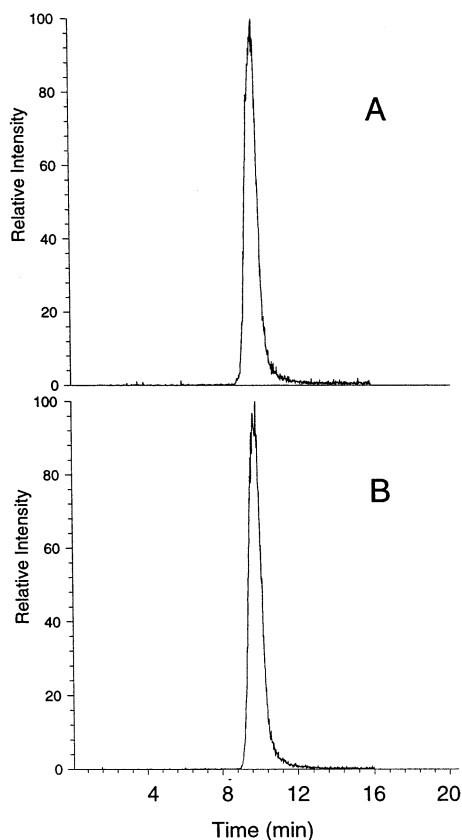


Fig. 3. SRM chromatograms of allantoin (A) and internal standard (B) in the day 2 urine sample of a subject treated with daily iv injection of urate oxidase. See text for other details.

Table 1

Precision and accuracy of within and between run analyses for allantoin in human urine

Nominal ($\mu\text{g ml}^{-1}$)	Within run ^a		Between run ^b	
	Precision ^c	Accuracy ^d	Precision ^c	Accuracy ^d
14.6	1.92	-2.74	3.55	0
23.1	1.02	-6.50	2.11	-6.64
113	0.784	-0.870	1.31	-0.870
213	1.47	3.26	1.36	1.37

^a Six replicates at each level.

^b Based on six runs, three replicates at each level in each run.

^c Coefficient of variance in percentage (CV%).

^d Mean percentage difference from nominal (M%D).

116) were utilized for the construction of calibration curve and quantitation.

2.3. Calibration

A control urine pool was used to prepare the calibration standards. The endogenous level of allantoin in the control urine was determined against a calibration curve prepared in water (see below for matrix evaluation). The endogenous concentration of allantoin plus that fortified gave rise to the nominal value. Urine calibration curves consisted of allantoin concentration levels of 14.6, 23.1, 63.1, 113, 163, and 213 $\mu\text{g ml}^{-1}$, with triplicates at both ends and singlets in between. A linear regression with $1/X^2$ weighting was used.

To demonstrate that the allantoin signal response was the same in human urine and in water,

Table 2

Urine (24 h pool) concentrations of allantoin from a subject during a daily iv infusion regimen of urate oxidase for 7 days

Day	Concentration ($\mu\text{g ml}^{-1}$)
Pre-dose	<MQL ^a
1	1398
2	1430
3	253
4	328
5	214
6	230
7	141

^a Less than minimum quantifiable level (14.6 $\mu\text{g ml}^{-1}$).

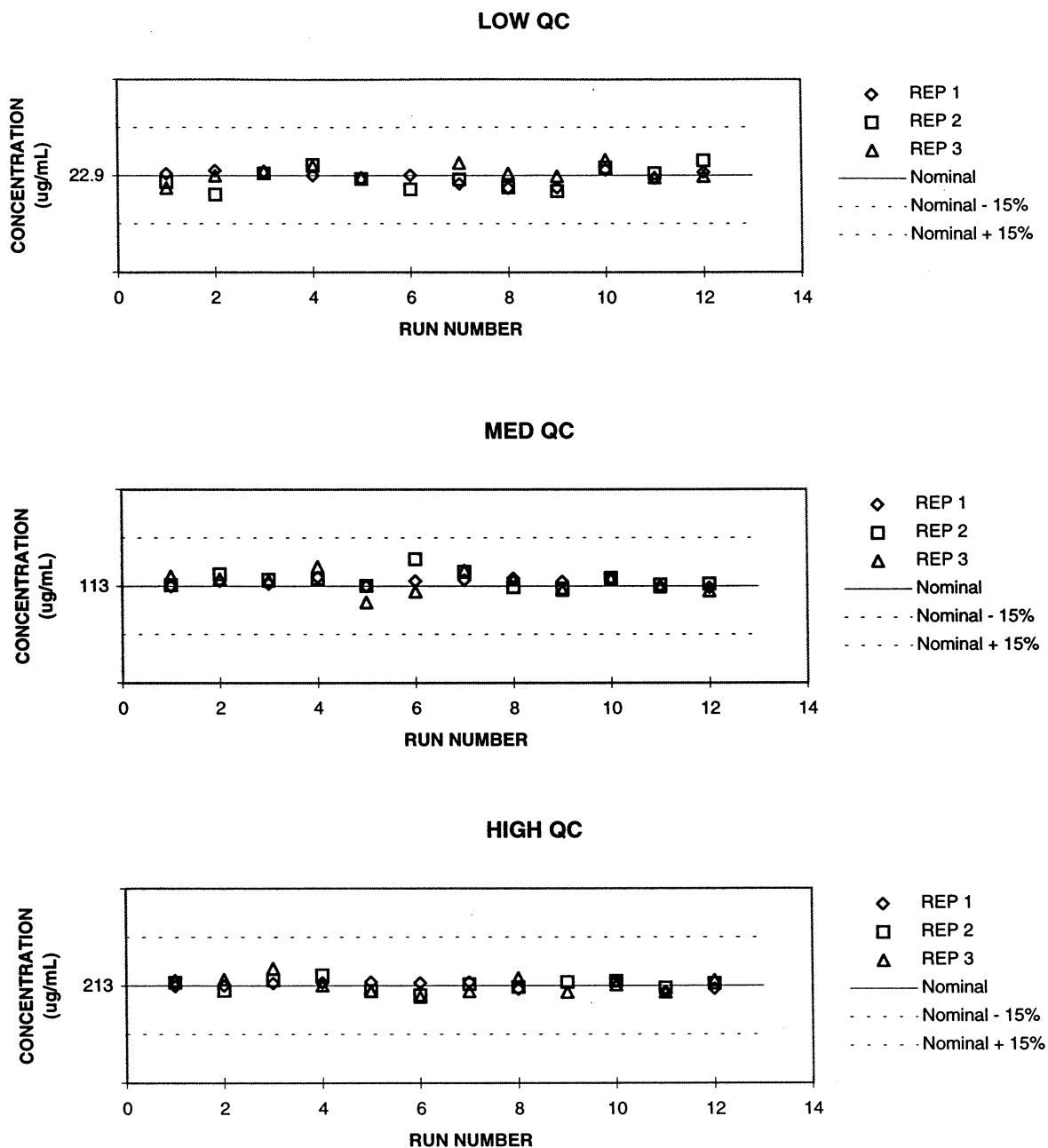


Fig. 4. QC control charts for clinical sample analysis.

the following experiment was carried out: a control urine containing $9.8 \mu\text{g ml}^{-1}$ endogenous allantoin (determined against a calibration curve in water) was fortified with allantoin to give final allantoin concentrations of 19.8 and $210 \mu\text{g ml}^{-1}$.

The allantoin fortified urine samples were then measured in six replicates at each concentration level against a water calibration curve. The coefficient of variance (CV%) and mean percentage difference from nominal (M%D) were all below

5%, indicating that there was no matrix difference between human urine and water under the experimental conditions employed. The control urine was used as the calibration matrix in this study to avoid potential matrix effects such as those induced by storage.

3. Results and discussion

Allantoin under the experimental conditions was detected in the Q1 scan predominantly as a parent ion $[M-H]^-$ at m/z 157 (Fig. 2). The major product ion at m/z 114 (Fig. 2) corresponding to the cleavage of formamide $[M-CONH_2]^-$ (Fig. 1) was used to monitor allantoin. SRM chromatograms obtained from the urine sample of a subject following an iv administration of urate oxidase are shown in Fig. 3

3.1. Precision and accuracy

Precision and accuracy were assessed based on within and between run analysis. For the within run analysis, six replicates were analyzed at each concentration level. For the between run analysis, three replicates were analyzed at each concentration level. A total of six runs were performed. The results are shown in Table 1. The coefficients of variance (CV%) and mean percentage differences from nominal (M%D) for all the analysis were below 7%.

3.2. Stability of allantoin during the sample preparation

The stability of allantoin in the sample preparation was assessed based on several aspects including: (1) freeze/thaw, in which urine samples fortified with allantoin were subjected to three cycles of freeze (-20°C) and thaw, followed by the measurement of allantoin; (2) post-sample process stability, in which the SPE processed samples were stored in the injection vials at room temperature (RT) for 72 h prior to the analysis by

LC-MS/MS; and (3) room temperature stability, in which the allantoin fortified urine samples were kept at room temperature for 24 h prior to sample preparation. Three concentration levels (14.6, 23.1, and $213\ \mu\text{g ml}^{-1}$) and six replicates at each concentration level were used for all the stability experiments. Allantoin was found to be stable in each of the sample preparation aspects examined (all CV% and M%D were below 6%).

3.3. Application to clinical sample analysis

The method has been successfully applied to the determination of urinary allantoin concentration levels in support of pharmacokinetic analysis for several clinical studies. The urine concentration of allantoin, based on the 24 h collection interval from a subject during a daily iv infusion regimen of urate oxidase for 7 days, is shown in Table 2. The assay performance in the clinical sample analysis was evaluated based on the QC control charts shown in Fig. 4. The difference from nominal was less than 13% for all the QC samples analyzed in 12 runs. In addition, statistics of the calibration curves was analyzed. The mean value of slope, intercept, and correlation coefficient based on the 12 runs was calculated to be 0.0158, 0.0574, and 0.998, respectively, the CV% was 3.75%, 57.9%, and 0.100%, respectively. The large CV% of intercept was a reflection of the instrument signal fluctuation.

References

- [1] J. Balcells, J.A. Guada, C. Castrillo, J. Gasa, J. Agric. Sci. 116 (1991) 309.
- [2] E.G. Young, C.F. Conway, J. Biol. Chem. 142 (1942) 839.
- [3] J. Balcells, J.A. Guada, J.M. Peiro, J. Chromatogr. 575 (1992) 153–157.
- [4] W. Tiemeyer, D. Giesecke, Anal. Biochem. 123 (1982) 11–13.
- [5] M.T. Diez, M.J. Arin, J.A. Resines, J. Liq. Chromatogr. 15 (1992) 1337–1350.
- [6] X.B. Chen, D.J. Kyle, E.R. Orskov, J. Chromatogr. 617 (1993) 241–247.