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Short communication

Separation and determination of allantoin, uric acid, hydantoin and urea

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Abstract

Hydantoin and urea, obtained by reducing the allantoin ring with hydroiodic acid or uric acid after treatment with uricase, were separated from each other and from their starting compounds by high-performance liquid chromatography and anion-exchange chromatography.

1. Introduction

Purine metabolism, followed by the incorporation of [^{14}C]formate into uric acid and allantoin in the rat liver, has long been used as an index of purine nucleotide catabolism [1–3]. The specific radioactivity of allantoin was found to be higher than that of uric acid a short time after administration of the precursor (17 and 30 min after administration of [^{14}C]formate). After 60 min, the values started to return to the same levels as for uric acid [4]. This quite unexpected result opens a new field in the study of purine catabolism.

One way to study this point and to explain these results is to measure the radioactivity of C-2 and C-8 in allantoin and uric acid. The procedure is based on the facts that: (1) during nucleotide catabolism, both compounds are selectively labeled in positions C-2 and C-8 when [^{14}C]formate is used as precursor; (2) purified uric acid is transformed in vitro into allantoin by treatment with uricase; (3) allantoin, however obtained, is transformed into hydantoin and urea by reductive hydrolysis with phosphonium iodide. The labeled carbons, originally in positions 8 and 2 of uric acid and allantoin, end up in hydantoin and urea, respectively (Fig. 1) The reduction of allantoin is reported in the literature [5] and involves the precipitation of hydantoin, but the working conditions used were not suitable for our study. Since we used about 0.1 mg of allantoin or uric acid, we could not obtain the hydantoin precipitate.

This made it necessary to use another specific procedure for the separation and determination of hydantoin, urea and allantoin; small amounts of allantoin were still present under our conditions and had to be separated from urea and hydantoin. The separation of these reaction products has never been reported in the literature. The three substances had a very similar chromatographic pattern and we could not use an ammonium buffer, because the urea determination was extremely sensitive to ammonium salts. We succeeded in separating hydantoin,

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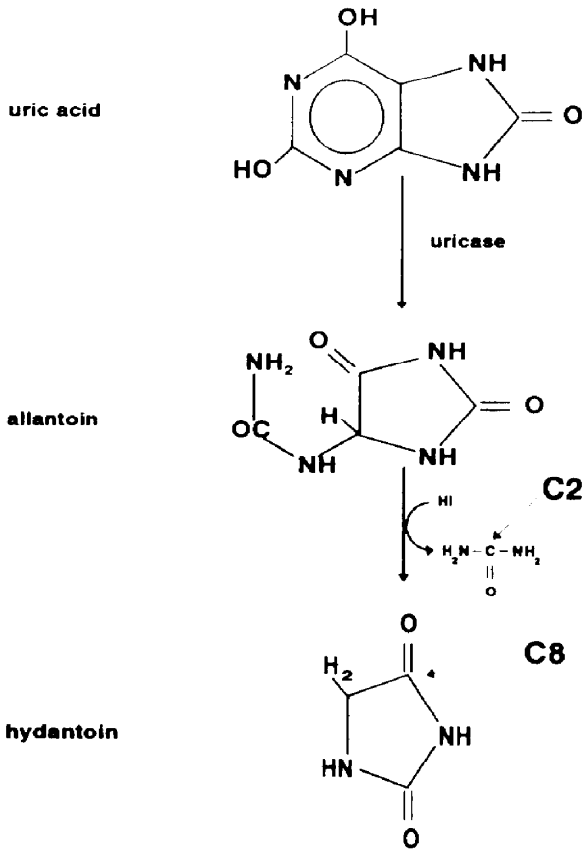

Fig. 1. Formation of hydantoin and urea.

urea and allantoin by HPLC and anion-exchange chromatography.

2. Experimental

2.1. Chemicals

Allantoin, uric acid, hydantoin and urea were purchased from Sigma (St. Louis, MO, USA). Uricase and urea stain were obtained from Boehringer Mannheim (Mannheim, Germany). Red phosphorus and hydroiodic acid were purchased from Fluka (Buchs, Switzerland). Anhydrous acetic acid was obtained from Farmitalia Carlo Erba (Milan, Italy). Methanol (HPLC grade) was obtained from Baker (Phillipsburg, NJ, USA).

2.2. Preparation of hydantoin and urea

We placed 0.1 mg of allantoin, 10 mg of red phosphorus, 0.1 ml of anhydrous acetic acid and 0.1 ml of hydroiodic acid, in a 1-ml vial. The mixture was refluxed for 4 h and filtered with cotton after cooling. The unreacted phosphorus was washed on the filter with two 0.05-ml portions of glacial acetic acid and discarded. The filtrate was applied to Dowex 1-X8 to remove all compounds used to reduce the allantoin. The column was equilibrated and then eluted with distilled water (flow-rate 0.2 ml/min). A 15-ml volume of eluate was collected. The mixture contained residual allantoin, urea and hydantoin, free of impurities. After neutralization, the solution was reduced in volume to 2 ml, using a rotary evaporation under vacuum, and separated by HPLC. Elution was carried out with distilled water pH 6.0 for 10 min at a flow-rate of 4 ml/min. In this way, hydantoin was separated from urea and allantoin. The urea–allantoin mixture was brought to pH 12 with NaOH, applied to an anion-exchange column (Dowex 1-X8) and eluted with distilled water (flow-rate 0.2 ml/min, fractions collected every 1.5 min). Fractions 13–27 contained only urea. Residual allantoin was eluted with 0.1 *M* NaOH.

Urea was determined by an enzymatic colorimetric method [6]. When treated with urease it produced ammonium carbonate which reacts with sodium hydroxide and hypochlorite to give a coloured complex (wavelength: 600 nm) (Fig. 2).

The same procedure was used for uric acid but 0.1 mg of compound was brought to pH 9.5 with NaOH and treated with uricase (12 μg) for 1 h to obtain allantoin [7]. The recovery of hydantoin and urea after the final step was about 70%.

Allantoin was assayed by the Rimini–Schryver reaction (see Ref. [8]).

2.3. Apparatus and chromatographic conditions

A Vista 5500 high-performance liquid chromatograph (Varian, Sunnyvale, CA, USA) equipped with a variable-wavelength UV detector (Model 2550, Varian) and an electronic inte-

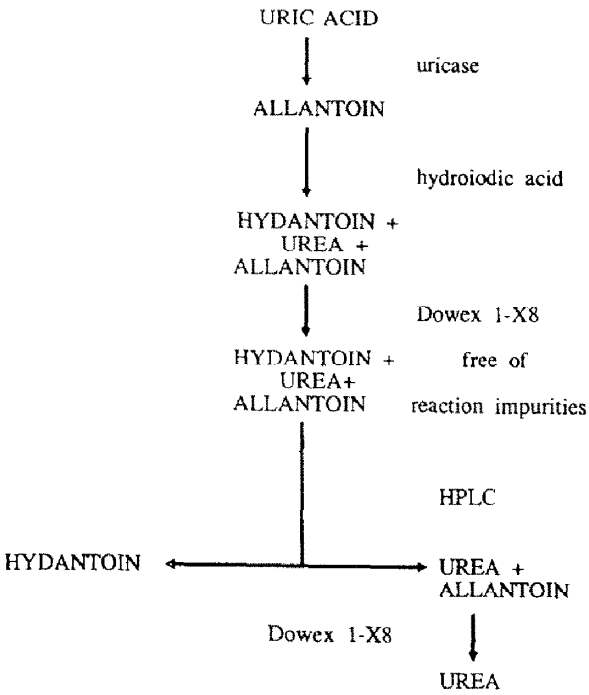

Fig. 2. Preparation and separation of hydantoin and urea.

grator (Model 4290, Varian) was used. A ready-for-use, prepacked Supelcosil LC-18 semipreparative column (250 × 10 mm I.D.), 5 μm (Supelco, Bellefonte, PA, USA) completed the analytical system. The mobile phase consisted of distilled water (pH 6.0), at a flow-rate of 4 ml/min. Detection was performed at 220 nm. Partisil 10 Sax (250 × 4.6 mm I.D.), 5 μm, was from Whatman, Supelcosil LC-SCX (250 × 4.6 mm I.D.), 5 μm, was from Supelco. A pH of 3 was obtained by acidifying distilled water with CF_3COOH; the pH of 6 was that of boiled distilled water; pH 3 and 6 were found to be constant during 10 min of HPLC analysis. Separation and retention times were the same using acidified water or 5 m*M* $NH_4H_2PO_4$ buffer.

A Dowex 1-X8 (200–400 mesh, anion-exchange resin) column (20 × 0.4 cm) was connected to an LKB Microperpex peristaltic pump and an LKB 2112 Redirac fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was equilibrated and eluted with distilled water at a flow-rate of 0.2 ml/min.

3. Results and discussion

Allantoin, urea and hydantoin were tested with three different types of chromatographic column (Table 1), but showed similar patterns when elution was carried out at pH 3 or 6, with water or buffer. Only with a LC-18 column we succeed in separating hydantoin from urea and allantoin. In our procedure, we prefer to use distilled water, since we had no trouble in detecting radioactivity. The compounds were detected at 220 nm. At this wavelength, urea did

Table 1
Retention times of hydantoin, urea and allantoin under different HPLC conditions

Compound	Retention time (min)		Column
	pH 3.0	pH 6.0	
Allantoin	3.50	3.60	Supelcosil LC-18
Urea	3.30	3.31	
Hydantoin	4.70	4.75	
Allantoin	3.37	3.38	Partisil SAX
Urea	3.47	3.49	
Hydantoin	3.38	3.41	
Allantoin	3.53	3.53	Supelcosil LC-SCX
Urea	3.48	3.48	
Hydantoin	3.85	3.85	

not show any significant absorption (molar absorption 0.03 $mmol^{-1}$ cm^{-1}). It can be detected at 200 nm (molar absorption 0.23 $mmol^{-1}$ cm^{-1}) and has a retention time of 3.3 min which is very similar to that of allantoin. The hydantoin peak eluted at 4.6 min and allantoin at 3.6 min (Fig. 3). Good linearity was obtained for all amounts of hydantoin and allantoin used (16–0.2 nmol). The correlation coefficients for hydantoin and allantoin were both 0.998 and the regression equations of the calibrations were $A = 68733C + 977$ and $A = 56777C + 8411$, respectively, where A = peak area and C (nmol) = amount of hydantoin or allantoin. The separation was reproducible column to column.

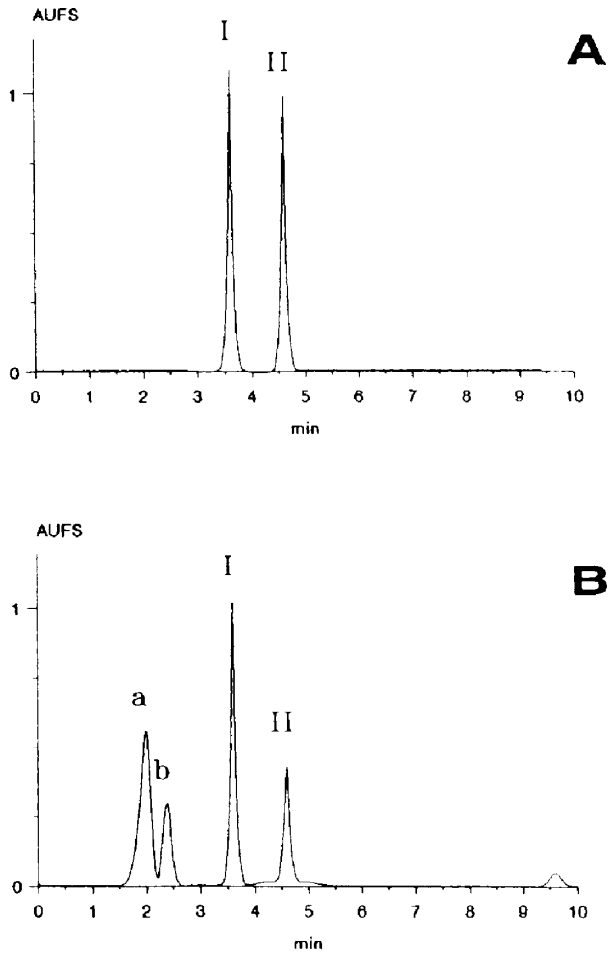

Fig. 3. (A) Separation of (I) allantoin and (II) hydantoin was carried out on a Supelcosil LC-18, with distilled water as mobile phase, at a flow-rate of 4 ml/min. Detection was performed at 220 nm. Injection volume: 20 μl of 1 mM solution of each standard. (B) Mixture containing reaction impurities (a, b), allantoin residues (I) and hydantoin (II). Urea is not visible because it is eluted at the same retention time as allantoin. Injection volume: 20 μl of solution diluted 1:20 with distilled water.

We obtained pure urea by applying the allantoin–urea mixture (pH 12) to the anion-exchange column (Dowex 1-X8) and collecting fractions 13–27. Separation of hydantoin and urea allowed the radioactivity of the labeled carbons to be measured. These were originally in positions 8 and 2 of uric acid and allantoin. Specific radioactivity was calculated.

3.1. Conclusions

The present method for the separation and determination of hydantoin, urea, allantoin and uric acid makes it possible to estimate, separately, the radioactivity of C-2 urea and C-8 hydantoin, obtained by reductive hydrolysis of allantoin with phosphonium iodide. The specific radioactivity of C-2 and C-8 obtained will be useful in the investigation of purine nucleotide catabolism and will help to explain why the incorporation of [^{14}C]formate into allantoin is much higher than into uric acid, a short time after administration of the labelled precursor in rat liver.

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