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Short communication

Separation and determination of liver uric acid and allantoin

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Abstract

We previously described the only satisfactory procedure yet achieved for separating uric acid and allantoin from rat liver. The procedure was based on trichloroacetic acid (TCA) extraction, acid hydrolysis, treatment with Hg-acetate, and cation- and anion-exchange chromatography. After separation, allantoin was quantified by a colorimetric method, and uric acid enzymatically using uricase. Since this procedure is too time-consuming, we propose an improved version which avoids the need for anion-exchange chromatography and the complex assay of catabolic compounds. The new method consists of a very fast and simple HPLC separation and direct determination of uric acid and allantoin at 220 nm. The method can be used for fresh tissue or after treatment of the tissue with labeled precursor.

1. Introduction

Uric acid and allantoin are the final products of purine catabolism in most mammals. It is widely accepted [1] that the quantity of these two compounds and the pattern of incorporation of radioactive label from precursors are two important parameters for the evaluation of purine catabolism. However, despite many attempts, accurate determination in tissue extracts and purification of these compounds for analysis of specific radioactivity are still problematical. Uric acid is generally determined enzymatically using uricase in trichloroacetic acid (TCA) extracts of tissues [2]. Satisfactory purification from rat liver has never been achieved and the total incorporated radioactivity has always been determined from paper chromatograms showing spots contaminated with glutamic acid [1]. Recently, several HPLC methods have been developed for the quantification of uric acid in serum [3], urine [4] and amniotic fluid [5]; very few procedures have been described for liver [6,7], and the effluents of perfused rat heart [8] and liver [9]. The Rimini–Schryver reaction is the only available method for determining allantoin in rat liver TCA extracts [10]; however, the reaction is not specific and is only valid for purified allantoin. In the past, the radioactivity of allantoin was determined in the same manner as for uric acid [1]. Later, it has been assayed by HPLC in plasma and urine [11], and in cosmetic and pharmaceutical products [12]. Recently it has been separated from uric acid as standards [13] and from biological fluids [14]. No separation has been reported from animal tissues, except for one analysis of rat liver perfused effluents [15].

In our previous study [16] uric acid and allantoin were obtained from rat liver and subjected to extensive purification by the following steps:

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TCA extraction (1 g of liver tissue + 5 ml 5% TCA), acid hydrolysis in 1 *M* H_2SO_4, Hg-acetate precipitation, cation-exchange separation of the catabolic compounds and the free purines, anion-exchange separation of uric acid and allantoin. The allantoin content was then measured by the Rimini–Schryver reaction as modified by Dietrich and Borries [10], and uric acid according to the method described by Praetorius and Poulsen [2]. Since this procedure is long and complicated, we devised a new method which avoids the need for anion-exchange separation and the subsequent determination of uric acid by the uricase reaction and allantoin by the Rimini–Schryver reaction. The present method uses a very fast and simple HPLC separation and direct determination of uric acid and allantoin at 220 nm.

A schematic representation of the old and the new procedure is shown in Fig.1.

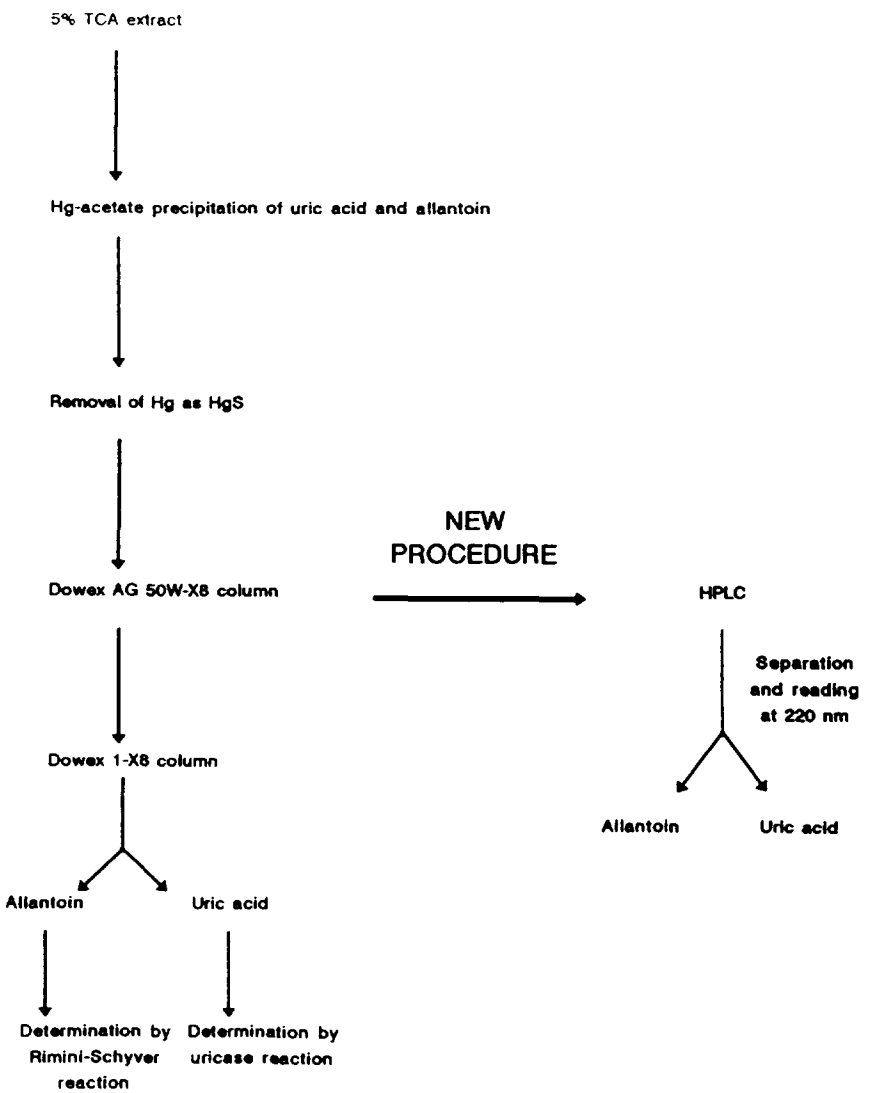


Fig. 1. Preparation of liver allantoin and uric acid.

2. Experimental

2.1. Chemicals

Allantoin and uric acid were purchased from Sigma (St. Louis, MO, USA). Ammonium dihydrogen phosphate, for use in HPLC, was obtained from Baker (Phillipsburg, NJ, USA). Uricase was supplied by Boehringer (Mannheim, Germany). All other products were of the highest commercially available purity.

2.2. Separation and purification of liver uric acid and allantoin

Male albino rats were decapitated and the livers immediately removed, washed in saline solution and homogenized at 5°C with 5% trichloroacetic acid (1 g of liver tissue + 5 ml TCA). The TCA extract was hydrolyzed in 1 *M* H_2SO_4 at 100°C for 1 h. After neutralization, allantoin, uric acid and free purine bases were precipitated by the addition of an equal volume of mercuric acetate solution (1 g mercuric acid and 10 g sodium acetate in 100 ml of distilled water). The precipitate was ground in 1 ml of distilled water and treated with hydrogen sulfide producing a large precipitate of mercuric sulfide. After removal of mercuric sulfide by centrifugation (15 min at 3000 *g*), the supernatant, containing the equivalent of 1 g tissue/ml, was applied on a Dowex AG 50 W-X8 column (10 × 0.4 cm I.D.; 200–400 mesh, cation-exchange resin), equilibrated with distilled water (flow-rate 0.5 ml/min; fractions collected every 2 min). Allantoin and uric acid were eluted with 20 ml of distilled water, concentrated to 0.5 ml and separated by HPLC. The equivalent of 400 mg of tissue can be analyzed in a single injection (200 μl).

2.3. Apparatus and chromatographic conditions

A Beckman (San Ramon, CA, USA) System Gold high-performance liquid chromatograph was used equipped with a Model 126 programmable solvent module and a Model 167 scanning detector module. A ready-for-use, prepacked (70 × 4.6 mm I.D.) Partisil 5 SAX, 5 μm column (Whatman, Clifton, NJ, USA), a strong anion-exchanger with quaternary nitrogen groups, completed the analytical system. The mobile phase consisted of 0.010 *M* ammonium dihydrogen phosphate buffer adjusted to pH 6.0 with NH_4OH, at a flow-rate of 1.0 ml/min. The eluate was detected at 220 nm.

Determination of uric acid

In the different purification steps, 50–200 μl of sample was mixed with 1 ml of 0.1 *M* sodium borate (pH 9.5), and 1 ml of this preparation was measured at 293 nm (initial absorbance = A_o). Then 10 μl of uricase (12 μg) was added and the decrease in absorbance was followed, every 5 min, for 30 min (final absorbance = A_f). The $A_o - A_f$ value was used to calculate the uric acid concentration, as previously reported [2].

Determination of allantoin

A 50–200 μl volume of sample was mixed with 160 μl of 12% TCA, 200 μl of 1 *M* NaOH and 280–130 μl of water and incubated for 7 min at 100°C. Then, 100 μl of a cold 1 *M* HCl solution and 100 μl of a O.33% phenylhydrazine solution were added, the mixture was boiled for 2 min and kept at −20°C for 3 min. Finally 300 μl of concentrated HCl and 100 μl of potassium ferricyanide (250 mg/15 ml of water) were added. The color was measured at 520 nm.

3. Results and discussion

During the chromatographic run, uric acid and allantoin were detected at 220 nm, since allantoin did not show any significant absorption spectrum (molar absorptivities at 220 nm of allantoin and uric acid are 1360 and 11 420 $mol^{-1}\ cm^{-1}$, respectively). The chromatographic behavior of allantoin did not change by varying the above chromatographic conditions in terms of pH or concentration of the eluent. Different pH values only modified the k' of uric acid. The effect of different mobile phase pH values in the separation of allantoin and uric acid is shown in Fig. 2. The pH chosen for the chromatography

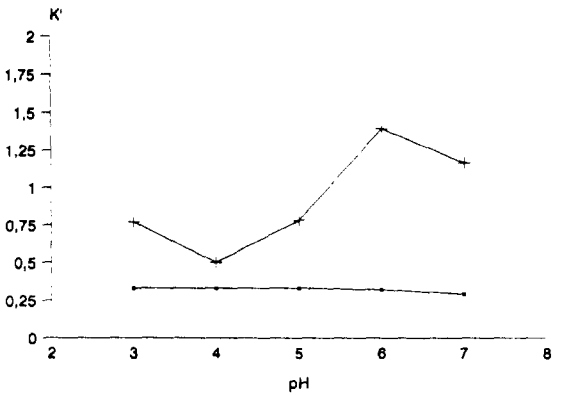
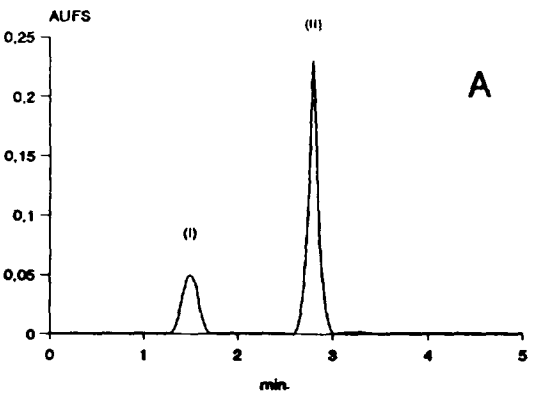


Fig. 2. Effect of pH on retention time of allantoin (■) and uric acid (+). $k' = (t_R - t_0)/t_0$, t_R = retention time of substance, t_0 = retention time of unretained solute.

separation was 6.0. Good linearity was obtained for allantoin concentrations in the range 0.1–150 nmol and for uric acid in the range 0.05–50 nmol.

The correlation coefficients for allantoin and uric acid were 0.975 and 0.976, respectively, and the regression equations of the calibration graphs were $y = 1.60x + 0.14$ and $y = 7.64x - 0.37$, respectively, where y is the peak area and x (nmol) is the amount of reagent. The overall between-run and between-day precisions of the retention times and peak areas are shown in Table 1. Fig. 3A shows a typical chromatogram of allantoin and uric acid in an artificially prepared solution. As seen here, satisfactory separation of the two substances was achieved under the chromatographic conditions used. Similar chromatograms



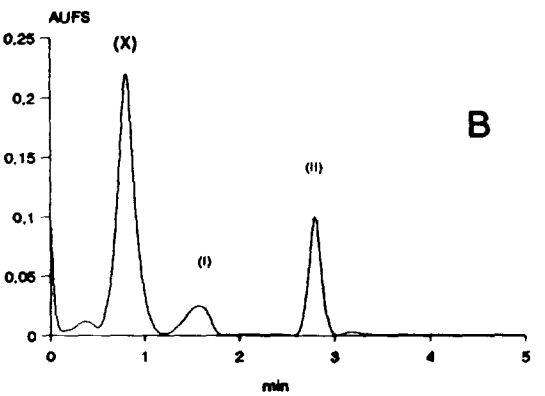


Fig. 3. (A) Separation of allantoin (I) and uric acid (II). Injection volume: 20 μl of 0.2 m*M* solution in each standard. (B) Allantoin (I) and uric acid (II) solutions prepared from rat livers by Hg-acetate precipitation and cation-exchange chromatography to separate them from the purine bases. (X) impurities from liver. Detection wavelength: 220 nm.

Table 1
Reproducibility and accuracy of retention times and peak areas of allantoin and uric acid

Compound	Retention time	S.D. ($n = 6$)	R.S.D. (%)	Peak area (arbitrary units)	S.D. ($n = 6$)	R.S.D. (%)
Between-run precision (over 1 day)						
Allantoin	1.54	0.004	0.26	3.27	0.04	1.22
Uric acid	2.74	0.010	0.36	15.14	0.21	1.35
Between-run precision (over 7 days)						
Allantoin	1.54	0.010	0.64	3.24	0.19	5.86
Uric acid	2.74	0.010	0.36	15.10	1.08	7.15

were obtained by injecting a solution of allantoin and uric acid prepared from rat liver, after Hg-acetate precipitation and elution on a cation-exchange resin to separate both catabolites from the purine bases (Fig. 3B). No contaminating compounds were found when the HPLC peaks were collected and analyzed in the UV region from 200 to 300 nm. The enzymatic determination of uric acid and the colorimetric assay of allantoin on the same peaks confirmed the quantity of both catabolites after HPLC. The true content of uric acid and allantoin was also determined by enzymatic and colorimetric assay after Hg-precipitation. The recovery of both compounds after cation-exchange purification and HPLC analysis was higher than 90%, while the recovery obtained after Dowex 1-X8 purification in the previous procedure was about 70%. When labeled compounds (after [^{14}C]formate injection in normal rats) were separated by the new procedure, the specific radioactivities of uric acid (5870 dpm/μmol) and allantoin (24 980 dpm/μmol) found 17 min after [^{14}C]formate administration were the same as those obtained by the previous procedure [16]. Because of the advantages of excellent resolution and short analysis time, the analytical method described here offers a convenient alternative to the previous method. Its simplicity and applicability to small sample volumes make it useful in biochemical research.

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