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Technical note

Automated determination of hypoxanthine and xanthine in urine by high-performance liquid chromatography with column switching

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Abstract

We report a high-performance liquid chromatographic method with column switching for urinary hypoxanthine and xanthine. Analyses were carried out with both a reversed-phase column and an anion-exchange column connected by a column switch and controlled automatically by a computerized system controller. The relationships between standard concentrations and peak heights were linear in a concentration range of 1 to 1000 nmol/ml. The recovery of hypoxanthine added to urine was 101.1%, and that of xanthine was 98.1%. With our method urinary hypoxanthine and xanthine can be measured accurately without any sample preparation other than filtration.

1. Introduction

The measurement of hypoxanthine and xanthine, which are intermediates in purine biosynthesis, is important for diagnosing purine metabolism disorders. Several high-performance liquid chromatographic (HPLC) methods for hypoxanthine and xanthine in serum have been reported [1–3]. However, HPLC assays of urine samples usually require a rather complicated sample preparation [4] due to the abundance of urinary compounds which interfere with hypoxanthine and xanthine measurements. We have established HPLC methods involving an automated column switching system for urinary pyrimidines, in order to measure pyrimidines accurately without sample preparation [5]. In this paper we describe a modified method for urinary hypoxanthine and xanthine.

2. Experimental

2.1. Chemicals and urine samples

Analytical-grade KH_2PO_4 and acetonitrile were purchased from Wako (Tokyo, Japan) and all other reagents were purchased from Sigma (St. Louis, MO, USA). Single voided urine samples were diluted 1:10 with deionized water and they were frozen and stored at −20°C. For sample preparation before HPLC analysis, only

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filtration was performed with a 0.45-μm Centricut filter (Kurabou, Osaka, Japan).

2.2. HPLC apparatus and procedure

Two Ryela PLC-5 liquid chromatographs (Tokyo Rikakikai, Tokyo, Japan) with a pump and detectors were used. An SC-15 computerized system controller (Tokyo Rikakikaki) consisting of an electric valve, a gradient system and a KSP-600 autosampler (Kyowa Seimitsu, Tokyo, Japan) was used. An on-line ERC-3611 Erma degasser (Erma, Tokyo, Japan) was used for eluent delivery, and a C-R4A Chromatopack integrator (Shimadzu, Kyoto, Japan) was used for data analysis.

A reversed-phase ODS column (MCI-ODS-1MU, 150 × 4.6 mm I.D., particle size 5 μm, Mitsubishi Kasei, Tokyo, Japan) was used as the first column. An anion-exchange column (MCI-GEL-SCA04, 150 × 4.6 mm I.D., particle size 5 μm, Mitsubishi Kasei) was used as the second column.

The ODS column was eluted for 10 min with 2 m*M* KH_2PO_4, adjusted to pH 4.8 with H_3PO_4, and then washed with acetonitrile–water (50:50, v/v) for 3 min. Finally, it was equilibrated with 2 m*M* KH_2PO_4 for 10 min. The anion-exchange column was eluted isocratically with 2 m*M* KH_2PO_4. The temperature of both columns was 40°C and both flow-rates were 0.8 ml/min. The retention times and peak heights were recorded using the C-R4A Chromatopack integrator. Each sample was applied to the first column, and fractions from the first column were delivered to the second column by an automated column switching system. Therefore, fractions which contained the target compounds flowed into the second column for accurate determination. The eluate from the second column was monitored at 265 nm, and 245 nm; values which are close to the maximum absorbance of xanthine and hypoxanthine. Each compound peak was identified on the basis of the retention time and the absorbance ratio, 265/245 nm. For further identification, we used the enzymatic peak-shift technique with xanthine oxidase [3]. The analytical recovery was studied by adding a 100 μl aliquot of a preanalyzed urine sample to obtain a concentration of 50 nmol/ml. Each sample was analysed five times.

3. Results and discussion

In a preliminary study we used only the first column (MCI-ODS-1MU). The retention time (mean ±S.D., $n = 5$) of the hypoxanthine standard was 8.23 ± 0.05 min, and that of the xanthine was 9.15 ± 0.06 min. Next, we included the column switching system. The fraction time for hypoxanthine ranged from 7.7 min to 8.8 min, and that for xanthine from 8.6 min to 9.8 min, according to the results of the preliminary study. The chromatographic profile of the hypoxanthine standard from the second column is shown in Fig. 1a, and that of xanthine is shown in Fig. 2a. The retention time (mean ±S.D., $n = 5$) of hypoxanthine was 11.3 ± 0.08 min and that of xanthine was 17.4 ± 0.08 min. The relationships between standard concentrations and peak heights were linear in a concentration range of 1 to 1000 nmol/ml. The correlation coefficients for hypoxanthine and xanthine under study (*r*, obtained from five measurements) were 0.999 and 0.997, respectively. The detection limit was 10

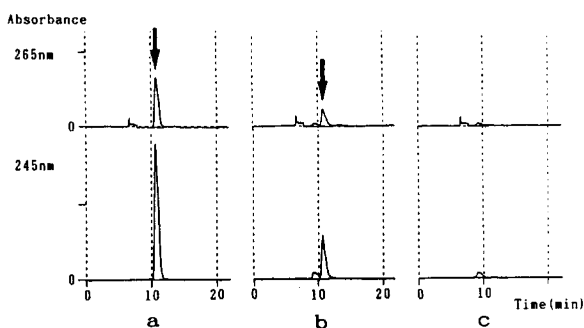

Fig. 1. Second-column chromatograms of hypoxanthine. Detection wavelength: (upper) 265 nm at 0.04 a.u.f.s.; (lower) 245 nm at 0.04 a.u.f.s. Chromatogram: a = standard mixture of hypoxanthine; b = 10-μl urine sample from a healthy adult; c = 10-μl urine sample, treated with xanthine oxidase. Arrows indicate the peaks of hypoxanthine. Small additional peaks at 265 nm are artifacts which are probably caused by column switching procedures.

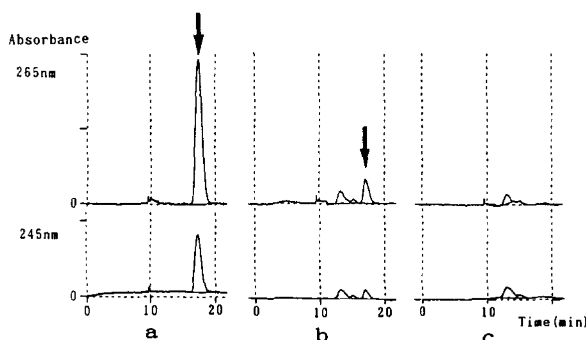

Fig. 2. Second-column chromatograms of xanthine. Detection wavelength: (upper) 265 nm at 0.04 a.u.f.s.; (lower) 245 nm at 0.04 a.u.f.s. Chromatogram: a = standard mixture of xanthine; b = 10-μl urine sample from a healthy adult; c = 10-μl urine sample, treated with xanthine oxidase. Arrows indicate the peaks of xanthine. Small additional peaks at 265 nm are artifacts which are probably caused by column switching procedures.

pmol per 10 μl injected in both cases. The absorbance ratio (mean±S.D., $n = 5$) of hypoxanthine was 0.378 ± 0.0034 and that of xanthine was 2.45 ± 0.036.

The chromatographic profiles of a urine sample are shown in Figs. 1b and 2b. The retention time and absorbance ratio of hypoxanthine were 11.1 min and 0.379, corresponding to those of the hypoxanthine standard. Those of xanthine were 17.3 min and 2.42, corresponding to those of the xanthine standard. The results for a urine sample treated with xanthine oxidase are shown in Figs. 1c and 2c. The peaks corresponding to hypoxanthine and xanthine disappeared completely, though small peaks from other urinary compounds remained. These results proved that the peak in Fig. 1b represented hypoxanthine and that in Fig. 2b represented xanthine. The recovery of hypoxanthine added to urine was 101.1%, and the coefficient of variation was 1.8%. The recovery of xanthine was 98.1%, and the coefficient of variation was 2.5%.

In healthy adults, preliminary reference values of hypoxanthine (mean±S.D., $n = 15$) and xanthine are 7.41 ± 3.58 μmol/mmol creatinine and 7.66 ± 6.27 μmol/mmol creatinine. These values correspond to those reported previously [4,6]. With our method, the total analysis time is only 30 min. More than 60 samples can be analysed consecutively. Because of the advantages of the excellent resolution and short analysis time, this method is useful clinically, not only for congenital purine metabolism disorders but also for other kinds of diseases, such as hyperuricemia.

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