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Determination of adenosine and deoxyadenosine in urine by high-performance liquid chromatography with column switching

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Abstract

The means of measurement of adenosine and deoxyadenosine in urine was developed by separating adenosine and deoxyadenosine from other compounds using high-performance liquid chromatography with column switchings. This method is simple and convenient since no pretreatment of the urine is needed. Using this method, it could be demonstrated that urinary adenosine was higher in an adenosine deaminase (ADA) deficient patient who had a bone marrow transplant treatment (1.97 μmol/mmol creatinine) and in a heterozygote who had a markedly low erythrocyte ADA activity (1% of control ADA activity) (1.33 μmol/mmol creatinine) as compared to normal subjects (0.22 ± 0.09 μmol/mmol creatinine, $n=11$). It was also noted that urinary deoxyadenosine was below the detection limits in the ADA-deficient bone marrow transplant patient, but it was detected in the heterozygote (3.7 μmol/mmol creatinine). Furthermore, it was also demonstrated that a fructose infusion increased the urinary concentration of adenosine from 0.21 ± 0.03 to 2.66 ± 1.21 μmol/mmol creatinine in five normal subjects.

Keywords: Adenosine; Deoxyadenosine; Adenosine deaminase; Fructose; Nucleosides

1. Introduction

Deoxyadenosine and adenosine are purine nucleosides which are important components of nucleic acid. They are metabolized to deoxyinosine and inosine, respectively, by adenosine deaminase (ADA). In patients with a deficiency of ADA, both substances increase in blood and urine. Increases in the intracellular concentrations of deoxyadenosine and adenosine disturb the metabolism of nucleic acid in cells, especially T and B lymphocytes, resulting in damage to the T and B cells which impairs the immunological function in ADA-deficient patients [1–4]. In previous studies [5,6], levels of deoxyadenosine and adenosine in urine were determined by high-performance liquid chromatography (HPLC) after treatment of the urine sample. However, this treatment is tedious and troublesome. Recently, with the improvement of the HPLC apparatus, many substances in urine can now be directly determined without sample pretreatment. Therefore, we attempted to measure deoxyadenosine and adenosine in urine without a sample pretreatment and developed an HPLC method for their measurement using two

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columns. Furthermore, using this method, the urinary adenosine level was determined in an ADA-deficient patient who had a bone marrow transplant (BMT) and in heterozygotes, to determine whether or not the urinary excretion of adenosine and deoxyadenosine increases. In addition, the urinary excretion of adenosine was measured by this HPLC apparatus before and during fructose infusion for the following reason.

Adenine nucleotide degradation (ATP→ADP→AMP→IMP→inosine→hypoxanthine→xanthine→uric acid) enhanced by fructose [7] increases the intracellular concentrations of ADP and AMP. Since AMP is dephosphorylated to adenosine by 5′-nucleotidase, an increase in the cellular concentration of AMP may accelerate the conversion of AMP to adenosine. If so, adenosine may be excessively produced by enhanced adenine nucleotide degradation and then excreted in urine. Therefore, in the present study, we also attempted to determine whether or not the urinary excretion of adenosine is increased by fructose.

2. Experimental

2.1. Chemicals

Adenosine, deoxyadenosine, purine nucleoside phosphorylase and adenosine deaminase were purchased from Sigma (St. Louis, MO, USA). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan).

2.2. Subjects and protocol

The study was conducted on five healthy males, aged from 35 to 49 years and weighing from 47 to 60 kg. The subjects had normal laboratory data. After informed consent was obtained, fructose (1 g/kg body mass) was infused over 1 h as a 10% solution after an overnight fast, except for water. Urine was completely voided 1 h before the beginning of the fructose infusion and then the urine was collected at an interval of 1 h, two times. Two weeks later, a control study was also conducted on the same subjects. The protocol was the same as in the study described above except using a 10% glucose solution instead of the 10% fructose solution.

2.3. Urine samples

In addition to the urine samples described above, we also used urine samples from 11 normal subjects, aged from 32 to 50 years, an ADA-deficient patient aged 7 years, and three heterozygotes (proposita's parents and grandfather). One of the heterozygotes presented a markedly low activity of erythrocyte ADA. However, the ADA-deficient patient presented a normal erythrocyte ADA activity as the patient had undergone BMT at the age of 6 years because of a severe case of combined immunodeficiency syndrome.

2.4. Apparatus and techniques

The chromatograph consisted of two CCPM pumps (Tosoh, Tokyo, Japan), a SC-8020 system controller (Tosoh), two spectrophotometric detectors (UV-8010 and UV-8020) (Tosoh) and a VC-8020 column switching valve (Tosoh). The chromatographic columns used were a Wakosil 5C18-200 (250×4.6 mm) (Wako Pure Chemicals) as the first column and Tosoh TSK Gel (ODS-120A) (250×4.6 mm) as the second column. In both columns, the mobile phase was 20 m*M* KH_2PO_4 (pH 2.2), the flow-rate was 1 ml/min and the detection wavelength was 254 nm. Ten to 40 μl of the urine sample was injected into the first column. The effluent from the first column passed the column switching valve and then an SC-8020 spectrophotometric detector where it was monitored. The dead volume between the column switching valve and the spectrophotometric detector was 0.2 ml. The retention time and width of the peak of adenosine was 17.6 min and 0.9 min, respectively, and was 21.4 min and 1.2 min, respectively, for deoxyadenosine. Therefore, in order to completely pass the fraction containing adenosine and the fraction containing deoxyadenosine to the second column with the switching valve, a sufficient amount of time was taken in addition to an adjustment of the time needed (0.2 min) for the dead volume to pass. As a result, the two columns were connected with the switching valve for the duration of time in which the fraction containing adenosine

was eluted (from 16.4 to 18.4 min) and the time in which the fraction containing deoxyadenosine was eluted (from 20.2 to 22.2 min) via the first column, and the effluent from the second column was monitored.

2.5. Assay of ADA activity in erythrocytes

The ADA activity of the erythrocyte was determined spectrophotometrically at room temperature using a reaction mixture containing 3.0 ml of 50 m*M* KH_2PO_4 (pH 7.4), 0.3 μmol adenosine, 0.04 U xanthine oxidase, 0.3 U purine nucleoside phosphorylase and 10 μl of 20% hemolysate [8].

2.6. Sample preparation

The urine was filtered with a chromatodisc 4A (pore size 0.2 μm) (Kurabo, Osaka, Japan) and used as a sample without dilution. To convert adenosine and deoxyadenosine in urine into inosine and deoxyinosine, respectively, adenosine deaminase (0.6 U) was added to 100 μl of urine from a heterozygote with a markedly low activity of ADA in erythrocytes and who excretes both adenosine and deoxyadenosine in urine.

2.7. Statistical analysis

Values are expressed as mean±S.D. The significance of the difference in the urinary excretion of adenosine before and after the beginning of the fructose infusion was assessed by the two-tailed paired Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Linearity and recovery of adenosine and deoxyadenosine

The linearity was tested on standards of adenosine and deoxyadenosine in an aqueous solution at various concentrations. The linearity was excellent up to 55 μ*M* for adenosine ($\gamma=1.0$, $P<0.01$), and up to 42 μ*M* for deoxyadenosine ($\gamma=1.0$, $P<0.01$). The regression lines were Y (concentration)$=0.157X$ (area)$+0.299$ and $Y=0.161X+0.349$, respectively. The lower detection limit of adenosine was 50 pmol and that of deoxyadenosine was also 50 pmol. Next, reproducibility and accuracy studies were performed on three concentrations of adenosine and deoxyadenosine and Table 1 shows the results. The recovery rates for adenosine and deoxyadenosine were 99.5 and 98.5%, respectively.

3.2. Chromatographs drawn by HPLC

Fig. 1A shows a chromatogram of the urine of the patient's mother which was obtained from the first column without the use of the column switching valve, and Fig. 1B shows a chromatogram with the use of the column switching valve. Fig. 2A shows a chromatogram of the patient's mother's urine which was obtained from the second column.

3.3. Identification of adenosine and deoxyadenosine in urine

The retention time and absorbance ratio were determined in order to identify adenosine and deoxyadenosine in urine. Any deviation from the retention

Table 1
Reproducibility and accuracy of adenosine and deoxyadenosine analysis in urine

Compound	Concentration added (μ*M*)	Concentration found (mean±S.D., $n=10$) (μ*M*)	Coefficient of variation (%)
Adenosine	4.05	4.01±0.05	1.2
	12.05	12.06±0.14	1.6
	24.05	23.95±0.29	1.2
Deoxyadenosine	5.02	5.01±0.07	1.4
	10.01	10.00±0.11	1.5
	20.03	20.01±0.32	1.6

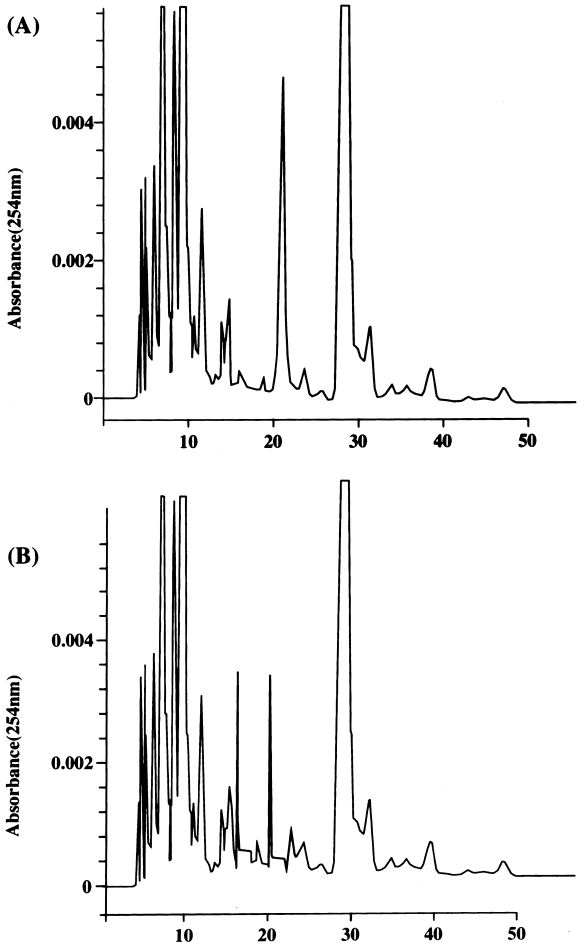

Fig. 1. First column HPLC chromatogram of 10 μl urine from a heterozygote with markedly low erythrocyte ADA activity. (A) Chromatogram without the use of the column switching valve. (B) Chromatogram with the use of the column switching valve.

time and absorbance ratio expected for adenosine or deoxyadenosine may indicate other compounds eluting near or simultaneously with them. The A_{270}/A_{254} ratios of the two peaks in the urine with the same retention time as adenosine and deoxyadenosine in a standard aqueous solution were 0.75 and 0.75, respectively. These values were identical to those for adenosine and deoxyadenosine. Furthermore, to 100 μl of the urine of the patient's mother was added 3 μl of adenosine deaminase which converts adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. The urine was incubated at 37°C for 1 h and its filtered sample was applied to the column. The peaks in the urine with the same retention time as adenosine and deoxyadenosine completely disappeared, indicating that the peaks in the urine showed adenosine and deoxyadenosine (Fig. 3).

3.4. Urinary excretion of adenosine and deoxyadenosine in normal subjects, ADA-deficient homozygote treated with BMT and heterozygotes

In the 11 normal subjects, urinary adenosine was 0.22±0.09 μmol/mmol creatinine (mean±S.D.) and urinary deoxyadenosine was below the detection limits. In the ADA-deficient patient treated with BMT, who had a normal erythrocyte ADA activity (1.05 μmol/gHb/min) (normal value: 0.8 to 1.55 μmol/gHb/min), urinary adenosine and deoxyadenosine were 1.97 μmol/mmol creatinine and below the detection limits, respectively. In the heterozygote with a markedly low erythrocyte ADA activity (0.009 μmol/gHb/min), they were 1.33 and 3.7 μmol/mmol creatinine, respectively. In the other two heterozygotes, whose erythrocyte ADA activities were 0.33 and 0.43 μmol/gHb/min, urinary adenosine was 0.3 and 0.28 μmol/mmol creatinine, respectively, and urinary deoxyadenosine was below the detection limits.

3.5. Effect of fructose infusion on the urinary excretion of adenosine

Fructose infusion increased the urinary concentration of adenosine from 0.21±0.03 to 2.66±1.21 μmol/mmol creatinine ($P<0.05$) in the five normal subjects (Fig. 3), while the glucose infusion did not change the concentration (data not shown).

4. Discussion

In previous studies [5,6,9–19], many analytical methods have been reported for measuring the concentrations of adenosine or deoxyadenosine in biological fluids by radioimmunoassay, fluorometric detection method, HPLC with fluorometric detection method or conventional HPLC with ultraviolet absorbance detection. Several of these methods are able to determine adenosine or deoxyadenosine in urine. Using these methods, the substances were measured

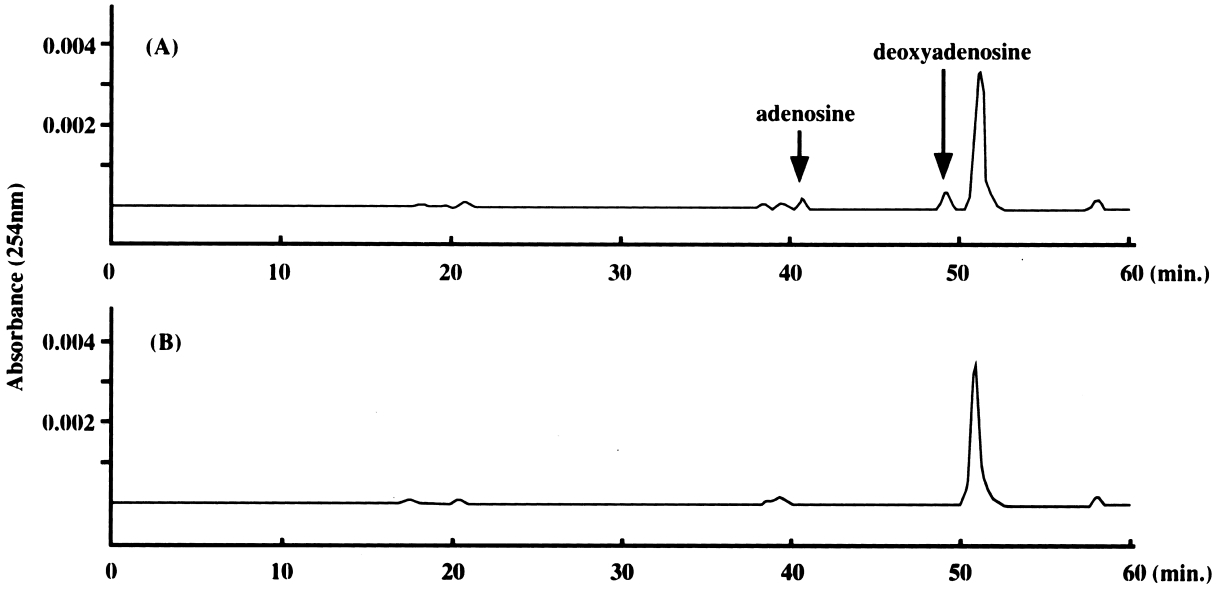

Fig. 2. Second column HPLC chromatogram of 10 μl urine from a heterozygote with markedly low erythrocyte ADA activity with the use of the column switching valve (A) before ADA treatment, (B) after ADA treatment. The urinary concentrations of adenosine and deoxyadenosine were 1.33 and 3.70 μ*M*, respectively.

in patients with ADA deficiency [6] and with malignancies [5]. However, since these analytical methods require a sample pretreatment such as nucleoside extraction from the biological fluids by a phenyl boronic acid column, they are tedious, and furthermore, need several ml of biological fluids for application onto the column. Therefore, we developed an HPLC method that requires no pretreatment to determine the concentrations of adenosine and deoxyadenosine in urine, one of the complex biological fluids. In the present study, two comparable C_{18} columns were used. Although the combination of two C_{18} columns seems to be illogical, a different characterization for each column made the clear separation of urinary adenosine and deoxyadenosine possible. In this method, the coefficient of variation (C.V.) and detection limits of adenosine were below 1.6% and 50 pmol and those of deoxyadenosine were also below 1.6% and 50 pmol. Although measurement methods for urinary deoxyadenosine and adenosine have been reported [6,20], no detailed data related to the C.V. of urinary deoxyadenosine have been described. A previous study demonstrated that the C.V. of urinary adenosine was 3% when using a boronate affinity column and HPLC [21]. Therefore, as for the C.V., our present method seems to be better than the previous one. In contrast, the sensitivity of adenosine and deoxyadenosine testing by our HPLC method seems to be slightly lower than that by the previous HPLC (those detection limits, 5 to 10 pmol) [5,11]. However, in urine, adenosine is present in sufficient quantity to be determined by our method and further, urinary deoxyadenosine is detected only in ADA-deficient homozygotes or in heterozygotes with a markedly low erythrocyte ADA activity whose urinary excretion of deoxyadenosine is sufficient enough to be determined by our method. Therefore, sensitivity does not seem to be a problem. The concentration of adenosine in the urine of normal subjects (μmol/mmol creatinine) determined by our new method is comparable with previously reported value [5], while that of deoxyadenosine was below the detection limits in normal subjects as described previously [22]. These results seem to indicate that this method is useful for determining the urinary concentration of adenosine and screening for ADA deficiency in spite of the 1 h analytical time because it is simple, convenient and does not need more than 40 μl of urine.

Previous studies [23–26] have demonstrated that

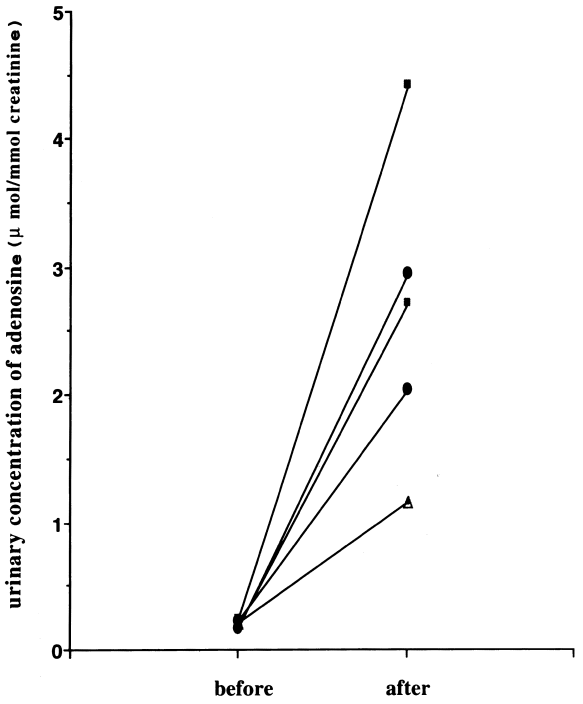

Fig. 3. Effect of fructose infusion on the urinary excretion of adenosine. Before, 1-h urinary excretion of adenosine prior to fructose infusion. After, 1-h urinary excretion of adenosine after the beginning of fructose infusion. Fructose infusion increased the urinary excretion of adenosine ($P<0.01$).

adenosine has several actions in the kidney such as a fall in glomerular filtration rate (GFR), alteration of the cortical distribution of blood flow and a decrease in renin release [23–26]. It has been further demonstrated that intrarenally produced adenosine plays a role in the intrinsic control of GFR, namely, increased production of adenosine in the kidney constricts the afferent arteriole and dilates the efferent arteriole thereby reducing glomerular capillary hydrostatic pressure and GFR. These results suggest that it is important to measure the renal concentration of adenosine, since it may regulate renal hemodynamics and natriuresis. Since adenosine excreted in urine may reflect adenosine produced by the kidney [25,26], its measurement seems to be a useful index of the renal concentration of adenosine.

In the present study, we measured urinary adenosine and deoxyadenosine in an ADA-deficient patient who underwent BMT and three heterozygotes. Two heterozygotes excreted adenosine in urine in the same levels as normal subjects. In contrast, the ADA-deficient patient treated with BMT and the other heterozygote, who had markedly low erythrocyte ADA activity, had an increased urinary excretion of adenosine, compared with that in normal subjects. However, the heterozygote also excreted deoxyadenosine in urine. Patients with ADA deficiency lack the activity of ADA in whole organs including the kidney, and as a result, excrete large amounts of adenosine and deoxyadenosine. The patient with the BMT also lacks the activity of ADA in whole organs except for the bone marrow and blood cells. Nevertheless, he did not excrete deoxyadenosine in urine (the same as normal subjects), although adenosine excretion increased. These results suggest that ADA-containing bone marrow and blood cells are most important in causing a decrease in the urinary excretion of deoxyadenosine. The urinary excretion of adenosine was high in the heterozygote with markedly low erythrocyte ADA activity and within the normal range in the other two heterozygotes as well as the normal subjects, suggesting that heterozygotes may not be detected by measuring the urinary excretion of adenosine. The urinary excretion of deoxyadenosine was found in the one heterozygote with markedly low erythrocyte ADA activity but no in the other two, suggesting that only heterozygotes with markedly low erythrocyte ADA activity can be detected by measuring the urinary excretion of deoxyadenosine. Therefore, the measurement of adenosine and deoxyadenosine in urine seems worthwhile only for the detection of ADA-deficient homozygotes and heterozygotes with markedly low erythrocyte ADA activity.

Fructose infusion increased the urinary excretion of adenosine (Fig. 3), indicating that enhanced adenine nucleotide degradation increased the production of adenosine, resulting in the increased urinary excretion. This suggests that the production of adenosine is increased by any substance which enhances adenine nucleotide degradation, such as fructose, xylitol, ethanol and exercise [7,27–30], and also, since the adenosine produced by these substances may act on the kidney, the effect of adenosine produced by these substances should be investigated in detail.

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