# A Simple Quantitative Assay for Urinary Adenosine Using Column-Switching High-Performance Liquid Chromatography

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TANIAI, H., SUMI, S., ITO, T., UETA, A., OHKUBO, Y. and TOGARI, H. A Simple Quantitative Assay for Urinary Adenosine Using Column-Switching High-Performance Liquid Chromatography. Tohoku J. Exp. Med., 2006, 208 (1), 57-63 — Adenosine is a physiologically active molecule produced locally in many sites of the body to regulate various cell functions. Measurement of levels of the factor in organs and biological fluids provides clues to its role and we reported an accurate quantitative high-performance liquid chromatography method for urinary adenosine requiring no preliminary sample preparation, other than filtration. Analyses were performed isocratically with a reversed-phase and a molecular exclusion columns connected by a column switch. Each sample was analyzed automatically in 35 min. Linearity could be verified up to 1,000  $\mu$ mol/L (r = 0.999) and recovery of adenosine was 94.6 - 98.0%. The coefficients of variation (CV) were established to be 0.56 - 1.32%, intra-assay, and 1.61 - 4.67%, inter-assay. Based on analyses of healthy individuals at different ages, we are here able to provide age-related values, infants  $(1.51 \pm$ 0.71  $\mu$ mol/mmol creatinine) and children (1.06 ± 0.36 and 0.83 ± 0.27  $\mu$ mol/mmol creatinine; aged 1 - 5 and 6 - 10 years), excreting significantly higher amounts of adenosine than adults (0.44  $\pm$  0.08  $\mu$ mol/mmol creatinine). We also measured urinary adenosine from patients suffering from metabolic disease or severe respiratory failure and found that unfavorable pathophysiologic conditions are associated with appreciable elevation of adenos-- adenosine; urine; HPLC; column-switching; metabolic disease ine. – © 2006 Tohoku University Medical Press

The sources of the purine and pyrimidine nucleotides are synthetic pathways and salvage pathways of exogenous and endogenous nucleobases and nucleosides. Defects in each of these metabolic pathways result in clinical disorders, including gout, the Lesch-Nyhan syndrome (McKusick 308000), orotic aciduria (McKusick 258900) and immunodeficiency conditions, for example, due to adenosine deaminase deficiency (Mckusick 102700) or purine nucleoside phos-

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phorylase deficiency (Mckusisk 164050). We earlier reported automated quantitative analyses for screening these diseases using highperformance liquid chromatography (HPLC) with column switching (Ohba et al. 1991; Sumi et al. 1995). Of the purine nucleosides, adenosine has been recognized for many years as an extracellular signaling molecule that can arise by breakdown of adenosine triphosphate (ATP) or by secretion from cells. Currently, it is clear that a number of adenosine receptor subtypes exist on the surfaces of human cells (LaNoue and Martin 1994; Miller and Hoffman 1994; Satoh et al. 1996; Porkka-Heiskanen et al. 1997). Adenosine works as a universal protective agent against hypoxia, ischemia, excitotoxicity, toxicities induced by other substances and trauma (Engler 1991). The measurement of urinary adenosine can contribute to evaluation of renal injury in various clinical settings (Katholi et al. 1995; Heyne et al. 2004). It is also an effective and safe therapeutic medicine for paroxysmal tachycardias in adult and pediatric patients, with basic electrophysiologic properties of slowing conduction in atrioventricular nodes (Ralston et al. 1994).

Several methods have been reported for the separation of purine nucleosides but require the use of either large volumes of body fluids, rather complicated procedures for preparation or expensive equipments. We have concentrated on development of a simple, rapid and reproducible method and here documented its use with urine samples from healthy individuals at different ages.

#### MATERIALS AND METHODS

#### Chemicals and solutions

Analytical-grade sulphuric acid  $(H_2SO_4)$  and acetonitrile were commercially acquired from Wako (Tokyo). Deionized water was passed through a Milli-Q Labo (Nihon Millipore Kogyo, Yonezawa). Adenosine was purchased from Wako as above.

#### Urine samples

Single voided urine samples were collected from healthy individuals (aged 0 year, n = 12; 1 - 5 years, n = 20; 6 - 10 years, n = 31; 11 - 15 years, n = 22; adults, n = 22; adults,

14), and from patients with adenine phosphoribosyltransferase deficiency (McKusick 102600), the Lesch-Nyhan syndrome, ornithine transcarbamylase deficiency (McKusick 311250) and orotic aciduria. Furthermore, we collected samples from individuals suffering from severe respiratory failure needing ventilatory assistance. All the urine samples were quickly frozen and stored at -20°C. Immediately prior to analysis, each sample was filtered through an ULTRAFREE-MC of 0.45 µm pore size (made by Millipore, Bedford, MA, USA) to remove particulate matter. All analyses were conducted using urine samples of 20  $\mu$ L. The urinary creatinine (Cr) levels were measured by an autoanalyser (TBA-30FR; Toshiba, Tokyo) following the Jaffe's method. Our study of human materials was approved by the ethical committee of Nagoya City University Graduate School of Medical Sciences.

### HPLC apparatus

The analyses were performed with an HPLC system consisting of two sets of an LC-10ATvp pump (Shimadzu, Kyoto), an LV-306R automatic high-pressure switching valve, an SIL-10ADvp auto sample injector and an SPD-M10Avp photodiode array UV-Vis detector for the second column (all from Shimadzu, Kyoto). A CLASS-LC10 system controller was used for data analysis (Shimadzu) and column temperature was maintained with a CT-10A column oven (Shimadzu). The HPLC apparatus configuration is illustrated in Fig. 1.

#### Columns and solutions

The first column was a reversed-phase ODS-C<sub>18</sub> column (Develosil ODS-HG-5, 150 mm × 6 mm I.D., particle size 5  $\mu$ m, Nomura Chemical, Seto), eluted with 5 mmol/L H<sub>2</sub>SO<sub>4</sub>. The second column was a molecular exclusion column (Asahipak GS-320HQ, 300 mm × 7.6 mm I.D., particle size 9 m, Asahi Seiko Kogyo, Tokyo), also eluted with 5 mmol/L H<sub>2</sub>SO<sub>4</sub>.

## HPLC procedure

A precisely measured 20  $\mu$ L aliquot of each sample was applied to the first column and one fraction (4.8 to 5.8 minutes after injection) containing adenosine was delivered to the second column by an automated columnswitching system. The first column was eluted with 5 mmol/L H<sub>2</sub>SO<sub>4</sub> for 10 minutes and was then washed with acetonitrile-water (50 : 50) for 2.5 min and the flow-rate was 0.8 ml/min. The second column was eluted isocratically with 5 mmol/L H<sub>2</sub>SO<sub>4</sub> and this flow rate was 1.0 ml/



Fig. 1. HPLC configuration: column 1, Develosil ODS-HG-5 (150 mm × 6 mm I.D.); eluent, 5 mmol/L  $H_2SO_4$ ; column 2, Asahipak GS-320HQ, (300 mm × 7.6 mm I.D.); eluent, 5 mmol/L  $H_2SO_4$ . With position A of the switching valve (SV) at 0 - 4.8 and 5.8 - 35 min, the two columns were separated. With position B at 4.8 - 5.8 min, the two columns were connected. The UV detector 1 in the dotted line box was applied to preliminarily monitor the eluate from the first column. All devices but the degasser were controlled by the CLASS-LC10 system.

min. Both columns were maintained at 40°C in a column oven. The eluate from the second column was continuously monitored at 255 and 270 nm, and peaks were detected with an SPD-M10Avp photodiode array UV-Vis detector. Retention times and peak heights were recorded using the CLASS-LC10 system controller.

### Statistical analyses

Statistical analysis of adenosine values in the samples was performed using the SPSS 13.0 J. Mean and s.D. values were calculated for the various groups. The "infants" group comprised twelve individuals aged under 1 year, the "children" group comprised seventy three individuals aged from 1 to 15 years, divided into five year sub-groups, and the "adults" group comprised fourteen individuals aged from 17 to 37 years. Differences between groups were analyzed with the Dunnett's pairwise multiple comparison t test and significance was set at p < 0.05.

#### RESULTS

## *Linearity and recovery*

The calibration curve for adenosine was

obtained by processing aliquots of an aqueous standard solution at different concentrations (0.5, 1, 5, 10, 50, 100, 500, and 1,000  $\mu$ mol/L; sample diluted with distilled water). The relationships between concentrations and peak heights were linear in the concentration range 0.5-1,000  $\mu$ mol/L, with a regression equation of: y =1000.4x - 279.53 (x in  $\mu$ mol/L; y in peak height).

 TABLE 1. Analytical recovery of adenosine
 added to urine sample<sup>a</sup>

Adenosir	Dagayany 0/-		
Added	Observed	Recovery, %	
10	$12.12 \pm 0.11^{b}$	94.6	
25	$27.88 \pm 0.54$	98.0	
50	$51.33 \pm 0.36$	97.1	
100	$99.92 \pm 2.63$	97.0	

<sup>a</sup> Analyses were done five times for each concentration.

<sup>b</sup> Mean ± s.D.

The correlation coefficient in this study (r, obtained from five measurements) was 0.999. The detection limit was 5 pmol in 10  $\mu$ L injected. The analytical recovery of the standard added to healthy adult urine is shown in Table 1 and was in the range of 94.6 - 98.0%.

## Precision

The methodological accuracy and precision

TABLE 2. Imprecision of Adenosine determination<sup>a</sup>

Sample	cv, %		
	Intra-assay	Inter-assay	
Standard (µmol/L)			
5	0.67	4.67	
10	0.89	2.83	
50	0.56	1.61	
Urine	1.32	2.73	

<sup>a</sup> Analyses were done six times for each sample.

were examined by repeated measurements of the standard and healthy adult urine on the same day (six repetitions) for intra-assay, and on six consecutive days for inter-assay (Table 2). The imprecision of the method (as CVs) for intra-assay at different concentrations of standard (5, 10, 50  $\mu$ mol/L) was 0.56 - 0.89 % and for inter-assay was 1.61 - 4.67%, whilst values for urine were 1.32% and 2.73%, respectively.

# Identification of adenosine in urine

For most nucleosides, peaks can be identified on the basis of retention time and chromatography of standards. We preliminarily examined whether separation of adenosine was achieved using the first column (ODS-C<sub>18</sub>) at 4.8 - 5.8 min, and the fraction was then delivered to the second column. The retention time of the adenosine standard monitored from the second column, which showed very little variation, was  $15.04 \pm 0.11$  min (mean  $\pm$  s.D. of five measurements). Fig. 2 shows a representative chromatogram of a urine sample and the standard. We confirmed the identity of



Fig. 2. Chromatograms and spectrograms of the adenosine standard and urine from a healthy child. Upper, chromatogram on the second column (one part from the first column at 4.8-5.8 min), monitoring at 255 nm; lower, spectrogram at the peak indicated by the arrow.

Aş (Ye	ge ear)	Cr (mmol/L)	Adenosine (µmol/L)	Adenosine (µmol/mmol Cr)
0	( <i>n</i> = 12)	$1.75 \pm 1.35$	$2.20 \pm 1.74$	$1.51 \pm 0.71^{*}$
1-5	( <i>n</i> = 20)	$5.88 \pm 3.39$	$6.58 \pm 4.48$	$1.06 \pm 0.36^{*}$
6-10	( <i>n</i> = 31)	$7.52 \pm 4.71$	$5.80 \pm 3.44$	$0.83 \pm 0.27^{*}$
11-15	( <i>n</i> = 22)	$11.69 \pm 5.44$	$6.63 \pm 4.18$	$0.56 \pm 0.21$
Adults	( <i>n</i> = 14)	$12.47 \pm 5.12$	$5.56 \pm 2.46$	$0.44 \pm 0.08$

TABLE 3. Adenosine in healthy individuals (mean  $\pm$  s.D.)

\* p < 0.05 in comparison to the adults group.

Cr, creatinine.

the peak by comparison with the spectrogram directly drawn by photodiode array detector for the standard (Fig. 2). Moreover, to verify the purity of the peaks, we monitored at two wavelengths, 255 and 270 nm, and established consistent peak height ratios for standard and urine samples of  $1.64 \pm 0.01$  and  $1.67 \pm 0.04$  (mean  $\pm$  s.d. of five measurements with coefficients of variation [CV] < 2%). Data for adenosine excretion in urine of healthy infants, children divided three subgroups, and adults are summarized in Table 3. In comparison to  $0.44 \pm 0.08 \ \mu \text{mol}/$ mmol Cr for the adult group, significantly greater adenosine excretion of  $1.51 \pm 0.71$ ,  $1.06 \pm 0.36$ and  $0.83 \pm 0.27 \ \mu \text{mol/mmol}$  Cr was seen in the infants, and the children groups aged 1 - 5 and 6 -10 years, respectively (p < 0.05).

 
 TABLE 4. Adenosine in metabolic disease and respiratory failure patients

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Patient		Age	Adenosine (µmol/mmol Cr)
	APRT deficiency	14 y.o.	0.43
	Lesch-Nyhan syndrome	10 y.o.	0.87
	OTC deficiency	9 y.o.	0.85
	Orotic aciduria	2 m.o.	1.15
	CMV pneumonia	3 y.o.	1.90
	Myocarditis	7 m.o.	3.61

APRT, adenine phosphoribosyltransferase; OTC, ornithine transcarbamylase; CMV, cytomegalovirus; Cr, creatinine; y.o., years old; m.o., months old. Regarding patients with severe respiratory failure needing ventilatory assistance, one suffered from severe pneumonia due to cytomegalovirus, and the other was in a critical condition with myocarditis. Their adenosine levels were 1.90 and 3.61  $\mu$ mol/mmol Cr, respectively. No one with inborn errors of purine and pyrimidine metabolism excreted excess amount of adenosine compared with normal subjects in the same age group (Table 4).

Fig. 3 shows adenosine concentrations plotted on the ordinate and the year after birth on the horizontal axis, the relationship being linear. The Pearson's correlation coefficient was -0.566 (p < 0.05) and the regression equation was y = -0.0281x + 1.150. Thus the value was age-dependent and over-excretion in infants and children can be concluded.

# DISCUSSION

The purpose of the present study was to establish a simple and quantitative assay for adenosine in human urine. Several HPLC methods have been reported, but these employed radioactive chemicals (Sato 1982), fluorescent materials (Fenton and Dobson 1987), and required preparation of derivatives with phenylboronate affinity columns, or protein precipitation (Davis et al. 1977; Agarwal et al. 1982; Echizen et al. 1989). Recently, a method for measurement of adenosine using reduced S-adenosylhomocysteine hydrolase was reported. This is a rather complicated procedure requiring expensive instrumentation (Kloor et al. 2000). They are intricate and need large



Fig. 3. Relation between the urinary excretion of adenosine and the year after birth. Open circles provide values observed in 99 healthy individuals and closed circles are for patients with inborn errors of purine and pyrimidine metabolism and suffering from respiratory failure.

volumes of biological fluids for application into the column. We here developed a clear separation using column switching. Our isocratic method eliminates the baseline drift that occurs with gradient elution, thus improving precision but maintaining high sensitivity. Moreover, we do not require any complicated pretreatment of samples other than filtration, so that our method enables savings in cost and time and avoidance of technical failure.

The concentrations and ratios to creatinine of urinary adenosine determined by our method are comparable with previously reported values (Hirschhorn et al. 1982; Sato et al. 1982; Katholi et al. 1995). Although values for small numbers of normal adult subjects have been published (Echizen et al. 1989), we analyzed a larger number and showed that children excrete significantly higher amounts of adenosine than adults in their urine. There is one report of analysis of purine and pyrimidine metabolites in children's urine (Vidotto et al. 2003) which showed excretion of higher amounts of these agents than adults, but the values for orotic acid, uracil and pseudouridine were 5 - 45 fold higher than with previous reports (Ohba et al. 1991; Asai et al. 2000). The purity and sensitivity is more valid in our method and the values for adenosine are reliable. We speculate that reduction of adenosine levels related to age may reflect fewer turnovers of nucleic acids after completion of the tissue modeling and development in children.

Determination of age related values showed infants and children to excrete significantly higher amounts of adenosine than adults. In addition, we analyzed urinary adenosine in patients with inborn errors of purine and pyrimidine metabolism and also cases with severe respiratory failure needing ventilator assistance and found 2-3 fold increase under such conditions. The physiological variability of urinary adenosine excretion is limited in humans and elevation thus reflects an unfavorable metabolic condition (Heyne et al. 2004). Therefore the values obtained for patients suffering from pneumonia and myocarditis catabolic situations resulting in a breakdown of cells, are of clear clinical relevance. Our present data suggest that excess excretion of urinary adenosine might result from disease conditions and especially with increased turnover of ATP under hypoxic

conditions (Ramos-Salazar and Baines 1986), in line with the earlier finding that augmented adenosine release from the breakdown of ATP is a natural defense against ischemia-reperfusion injury through several mechanisms, including vasodilatation, counteracting adrenergic stimulation (Engler 1991).

In conclusion, the method described above for determination of quantity of urinary adenosine is not only more convenient to perform but also requires less time, financial input and sample amount than reported for other approaches. Moreover, we believe that further accumulation of data should allow sophisticated reference agerelated ranges of adenosine in urine to be generated.

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