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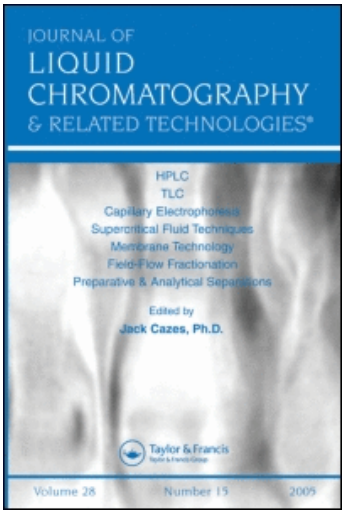

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ANALYSIS OF ADENOSINE AND OTHER ADENINE COMPOUNDS IN PATIENTS WITH IMMUNODEFICIENCY DISEASES

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ABSTRACT

Adenine compounds can be measured in picomole amounts using liquid chromatography of the fluorescent 1,N^6-etheno derivatives. The limit of detection for the etheno derivatives in tissue extracts, however, is tissue-dependent due to interference by nucleotides and fluorescent components which are normally present. Prior to derivatization nucleotides were partially removed from extracts of lymphocytes and erythrocytes by treatment with Dowex AG1-X2 anion exchange resin. Samples were analyzed using either a Partisil PXS 10/25 SCX column eluted with 100 mM $NH_4H_2PO_4$, pH 4.5, at a flow rate of 2 ml/min; or using two μBondapak/C_{18} reversed-phase columns eluted with 5 mM KH_2PO_4:25% methanol (V/V) pH 7.5, at a flow rate of 1 ml/min. Adenosine was found to be 0.07 nmole/ml in normal adult human plasma. The urine of a child with severe combined immunodeficiency disease associated with absence of adenosine deaminase contained a normal amount of adenosine (5-6 nmole/ml), but contained a high level (~60 nmole/ml) of deoxyadenosine. Deoxyadenosine was not detected (<0.01 nmole/ml) in normal adult urine. Because of its sensitivity and selectivity, this method of analysis should be applicable to studies of the physiological roles of adenine compounds.

INTRODUCTION

Adenosine (1) is a substance which has profound effects on several physiological systems. These include coronary and cerebral circulation (2), cyclic AMP regulation (3), neurotransmission (4) and platelet aggregation (5). Recently, adenosine has been

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implicated as a possible causal agent in severe combined immunodeficiency disease characterized by absence of adenosine deaminase (6). In the presence of adenosine deaminase inhibitors, adenosine exerts growth-inhibitory effects and impairs lymphocyte function (7). To better understand the roles of adenosine in the above systems, a method for its analysis has been developed and is reported here. The method utilizes fluorescent detection of the $1,N^6$-etheno derivative following separation by liquid chromatography. A preliminary report of this work has been presented (8).

MATERIALS AND METHODS

Biological procedures. Samples of blood and urine were obtained from a child (age, 30 months) with severe combined immunodeficiency:adenosine deaminase deficiency (SCID:ADA$^-$). This is an autosomal recessive disorder and is characterized by profound deficiency and dysfunction of both humoral and cell-mediated immunity. During the period of this study, the child was treated with erythrocyte transfusions (9). A urine sample was obtained from a child (age, 30 months) with Wiskott-Aldrich Syndrome, an x-linked recessive disorder with an associated immunodeficiency state (10).

Urine samples from four normal adults and the immunodeficient children were initially prepared using a 1 x 5 cm column packed with analytical grade Dowex AG1-X2 (200/400 mesh) anion-exchange resin obtained from Bio-Rad Laboratories. Adenine compounds were separated using two successive elution conditions. Initially, the anion exchange column was equilibrated with 20 ml of 1.5 mM NH_4OH, 0.1 M NaCl, pH 10. Three ml of urine were diluted to a volume of 10 ml using deionized water and adjusted to pH 10 using 1 N NaOH. The urine was washed through the column using 45 ml of deionized water. This 55 ml fraction contained adenosine and deoxyadenosine. Adenine was collected by washing the anion exchange column with 10 ml of 77 mM sodium acetate buffer, pH 5.25, followed by 10 ml of deionized water. Recoveries were determined using standard solutions treated similarly.

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Heparinized venous blood samples were obtained from six normal adults and from the SCID:ADA$^-$ child. Erythrocytes and plasma were separated by centrifugation and the plasma was carefully removed. The packed erythrocytes and plasma were deproteinized using equal volumes of cold 12% trichloroacetic acid solution. After incubation at 4^o for 5 min, the samples were centrifuged and the supernate was collected. Trichloroacetic acid was removed with four volumes of water-saturated ether.

Lymphocytes were isolated from six normal individuals and from the SCID:ADA$^-$ patient using Ficoll-Hypaque density centrifugation as described by Bøyum (11). After centrifugation at 2,000 xg for 10 min, the lymphocyte-platelet layer was collected. The cells were washed three times in Hank's balanced salt solution and suspended in phosphate-buffered saline. Approximately 10^7 lymphocytes were collected from each of the normals and about 10^6 lymphocytes were collected from the SCID:ADA$^-$ patient. Deproteinization was performed using cold 12% trichloroacetic acid as described above.

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Nucleotides were removed from the erythrocyte and lymphocyte extracts with an anion exchange resin prior to derivatization. A volume of 0.2 ml of a thick slurry of Dowex AG1-X2 (200/400 mesh), which was equilibrated with 0.1 M sodium acetate buffer pH 5.5, was added to the sample extracts. After a five min incubation at room temperature, the anion exchange resin was removed by filtration through 0.45μ filters (Millipore Corp., Bedford, MA). Recoveries were determined using {8-^{14}C} -adenosine (54.7 μCi/μmole, New England Nuclear, Boston, MA) and {8-^{14}C} -adenine (46.9 μCi/μmole, Schwarz-Mann, Orangeburg, NY).

Sample derivatization. For preparation of the 1,N^6-etheno derivatives (12), the procedure reported by Mills et al. (13) was used. Chloroacetaldehyde was prepared from chloroacetaldehyde dimethyl acetal (13, Aldrich Chem. Corp., Milwaukee, WI). Samples (0.5 to 1.0 ml) were buffered to pH 5 using 0.1 M sodium acetate buffer and chloroacetaldehyde was added to a final concentration of 67 mM. The mixture was incubated at 80^o for 40 min and the excess

chloroacetaldehyde was removed by extraction with water-saturated ether. Sample blanks without chloroacetaldehyde were prepared simultaneously. All samples were stored at -20° until assayed.

Liquid chromatography. Biological samples and standard solutions were assayed using a Waters Associates (Milford, MA) Model 204 Liquid Chromatograph. Fluorescence was monitored with a Schoeffel Instruments (Westwood, NJ) Model FS-970 Fluorescence Detector and the response was recorded on a Houston Instruments (Austin, TX) Chart Recorder, Model A5211-1. An excitation wavelength of 280 nm with excitation filter No. 7-54 and emission filter KV-389 were used. The sensitivity setting was 63.0 and the time constant was four seconds. The range setting is given in the figure legends and lower range settings reflect higher sensitivities. Either of two different chromatographic systems was used. Two μBondapak/C_{18} (4 mm x 30 cm) reversed-phase columns (Waters Associates) connected in series with minimal dead-volume fittings were eluted with 5 mM KH_2PO_4:25% methanol (V/V), pH 7.5, at a flow rate of 1 ml/min. A Partisil PXS 10/25 SCX column (4 mm x 25 cm; Whatman, Inc., Clifton, NJ) was eluted with 100 mM $NH_4H_2PO_4$, pH 4.5, at a flow rate of 2 ml/min.

Concentrations of adenine compounds in the biological samples were determined by comparing peak areas (measured as peak height times peak width at one-half peak height) to those obtained with authentic 1,N^6-ethenoadenosine or with etheno derivatives prepared from adenosine, deoxyadenosine, adenine, cyclic adenosine-3',5'-monophosphate, tubercidin or adenosine-5'-monophosphate (P-L Biochemicals, Milwaukee, WI). Adenine compounds in biological samples were identified by: 1) comparison of retention times with standards; 2) reaction with chloroacetaldehyde to form fluorescent derivatives; 3) sensitivity to acid hydrolysis; and 4) where indicated, sensitivity to adenosine deaminase (EC 3.5.4.4, calf intestinal enzyme, Sigma Chemical Co., St. Louis, MO). The recovery after purification and derivatization of adenosine and deoxyadenosine in urine samples was estimated to be 60% and the recovery of adenine was 75%. Using

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bulk anion exchange to remove nucleotides from erythrocytes and lymphocytes resulted in a 58% recovery of{8-^{14}C}-adenosine and a 34% recovery of {8-^{14}C}-adenine. All reported values were corrected accordingly.

RESULTS

Separations of standard solutions of 1,N^6-etheno derivatives of adenine compounds are illustrated in Figure 1. Either a Partisil PXS 10/25 SCX column (A) or two C_{18} μBondapak reversed-phase columns (B) were used as described in Materials and Methods. In the range of 0 to 50 picomoles of injected sample, the fluorescent detector response is linear for the etheno derivatives (Figure 2). The reduced response for adenine using the cation exchange column (A) was due to the lower pH used (14). Approximate lower limits of detection for adenosine and adenine derivatives under these conditions were one and five picomoles, respectively.

Adenosine was detectable as its 1,N^6-etheno derivative in normal human urine (Figure 3). The urine of the SCID:ADA$^-$ patient contains adenosine, and also contains large amounts of deoxyadenosine. Identity of these components was supported by the observation that treatment with adenosine deaminase prior to derivatization eliminates the peaks. A summary of the levels of adenine compounds detected in urine by this procedure is given in Table 1. The results given are mean values± S.D. for four normal adults or are single determinations on samples from the affected children. The level of adenine in the urine from the SCID:ADA$^-$ patient "after transfusion" was not determined in these samples.

The adenine and adenosine concentrations were elevated in the lymphocytes of the SCID:ADA$^-$ patient (Table 2). The lower limit of detection of deoxyadenosine was estimated to be 25 nmoles/ 10^9 cells in these extracts due to interference by an unidentified compound which was also present in erythrocyte extracts. The level of detection was estimated by adding known amounts of authentic 1,N^6-ethenodeoxyadenosine to the extract prior to liquid chromato-

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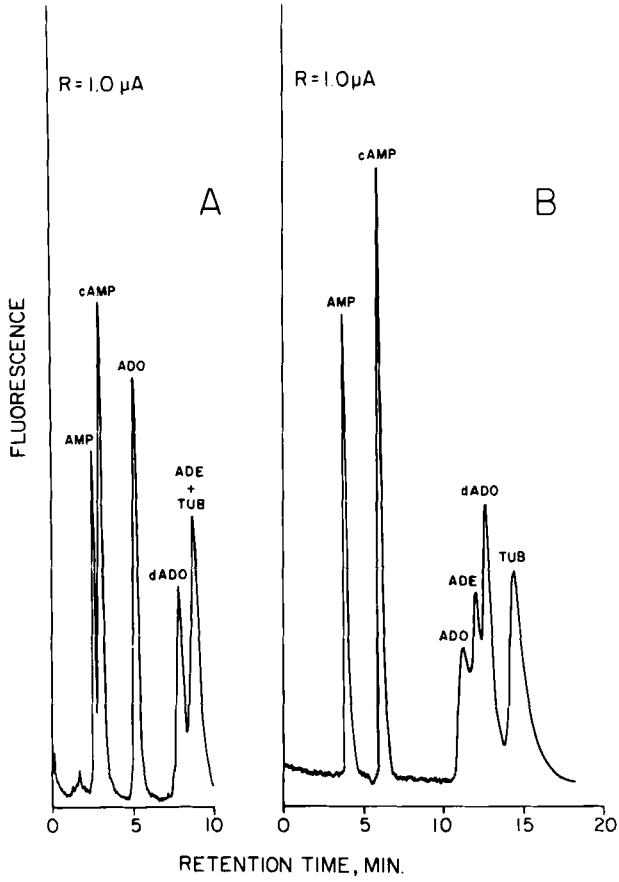

FIGURE 1

Representative separations of 1,N^6-etheno derivatives of adenine compounds using either (A) a Partisil PXS 10/25 SCX column eluted with 100 mM $NH_4H_2PO_4$, pH 4.5, at a flow rate of 2 ml/min; or (B) two μBondapak/C_{18} reversed-phase columns eluted with 5 mM KH_2PO_4:25% methanol (V/V), pH 7.5, at a flow rate of 1 ml/min. The amount of material injected was 20 picomoles of each compound in a total volume of 20 μl. R = Range.

graphy. The level of adenosine in normal, adult human plasma was found to be 0.07±0.03 nmoles/ml (mean± S.D., n = 6; Table 3). Deoxyadenosine levels were less than 0.06 nmoles/ml (calculated

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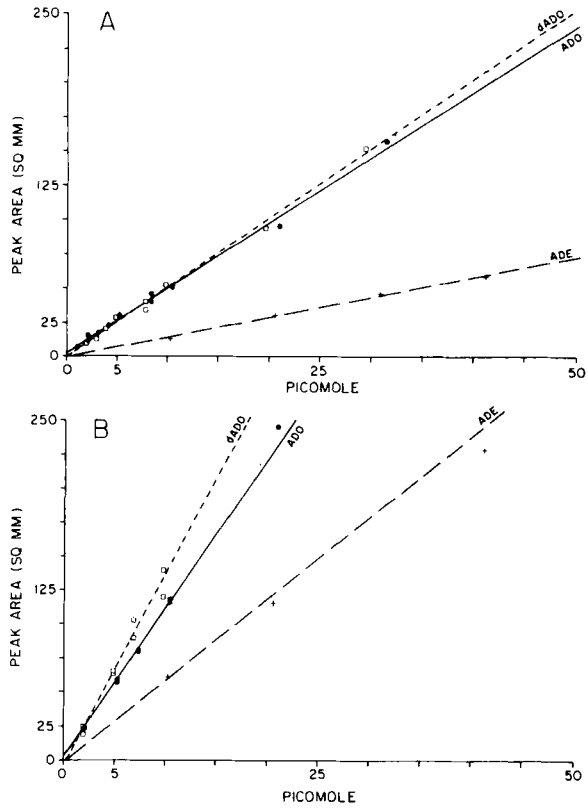

FIGURE 2

Fluorescence detection of adenine components as their 1,N^6-etheno derivatives. Detector response to authentic 1,N^6-etheno derivatives of adenine (+), deoxyadenosine (■), and adenosine (●) was determined from separations using either (A) the Partisil SCX column or (B) the two μBondapak/C_{18} columns (see Figure 1). Range = 1.0 μA

minimal detectable amount). In samples taken three or more times before and after transfusion of the SCID:ADA^- patient, the level of adenosine in plasma varied between 0.1 to 2.0 nmoles/ml. This variation has been observed in a number of different transfusions and is related to the time following red blood cell transfusions(9).

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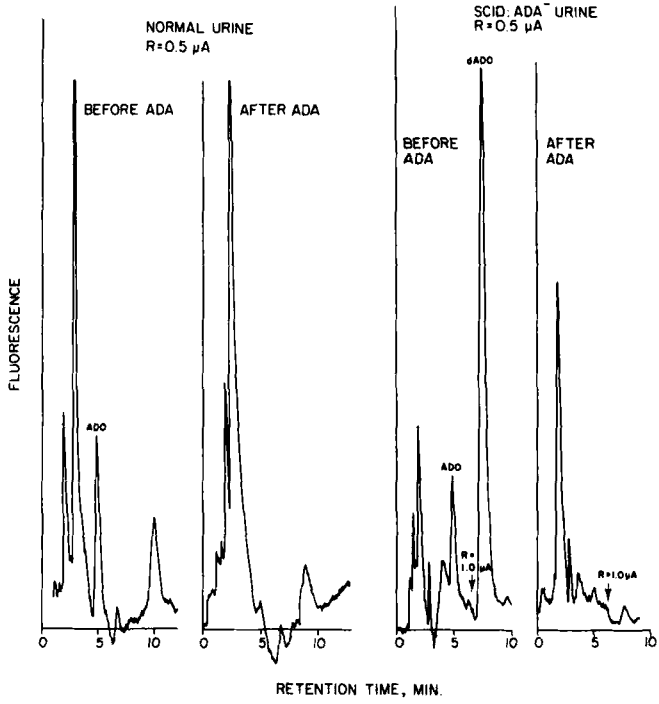

FIGURE 3

Representative chromatograms of human urine illustrating adenine compounds in a normal individual and the SCID:ADA⁻ patient. 1,N^6-etheno derivatization was performed without prior treatment of the extracts with adenosine deaminase (before ADA) or after treatment of the extracts with adenosine deaminase (after ADA). The injection volume (50 μl) was equivalent to 3 μl of urine. The Partisil SCX column was used. R = Range.

Adenosine levels in red blood cells taken from six normal adults averaged 0.20 ± 0.05 (mean±S.D.) whereas adenine was about 16 nmoles/ml (Table 4). Deoxyadenosine was not detectable (less than 0.2 nmoles/ml). As previously reported (13), adenosine and adenine were elevated in erythrocytes of the SCID:ADA⁻ patient.

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TABLE 1

Levels (nmoles/ml) of adenine compounds in human urine

	ADO	dADO	ADE
Normal	5.4±4.5	<0.01	3.2±2.3
SCID:ADA$^-$			
Before transfusion	5.6	68	50
After transfusion	5.3	25	--
WISKOTT-ALDRICH	6.9	<0.01	<0.02

TABLE 2

Levels (nmoles/10^9 cells) of adenine compounds in human lymphocytes

	ADO	dADO	ADE
Normal	29±15	<25	129±32
SCID:ADA$^-$			
Before transfusion	55	<25	488
After transfusion	42	<25	297

TABLE 3

Levels (nmoles/ml) of adenine compounds in plasma

	ADO	dADO
Normal	0.07± 0.03	< 0.06
SCID:ADA$^-$		
Before transfusion	0.93± 0.80	< 0.06
After transfusion	0.42± 0.46	< 0.06

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TABLE 4

Levels (nmoles/ml) of adenine compounds in human erythrocytes

	ADO	dADO	ADE
Normal	0.20 ± 0.05	< 0.2	16 ± 7
SCID:ADA$^-$			
Before transfusion	0.68 ± 0.45	< 0.2	95 ± 13
After transfusion	0.41 ± 0.11	< 0.2	42 ± 8

DISCUSSION

Analysis of adenine compounds in the picomole range is possible following separation of the fluorescent 1,N^6-etheno derivatives (Figure 1). Yoshioka and Tamura (15) as well as Nelson (16) recently suggested this mode of analysis. This current report extends these observations and confirms the applicability to biological systems.

Previous methods for analysis of adenosine have utilized adenosine deaminase (17) or adenosine kinase (18). These methods suffer from either lack of sensitivity and selectivity (deaminase) or lack of availability and stability of the enzyme (kinase). The adenosine kinase method has recently been used to monitor this purine in brain (19) and other tissues (18). Analysis of adenosine using liquid chromatography with conventional ultraviolet absorbance detectors suffers from lack of sufficient sensitivity to easily measure the levels of adenosine normally present in biological tissue (20).

The high level of deoxyadenosine observed in the urine of the SCID:ADA$^-$ patient is consistent with the recent report of Cohen, et al. (21) in which deoxyinosine was observed in the urine of a patient lacking purine nucleoside phosphorylase. It is likely that the deoxyinosine resulted from deamination of deoxyadenosine in their patient. In the SCID:ADA$^-$ patient, deoxy-

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adenosine is excreted in the urine since adenosine deaminase is absent. Absence of detectable quantities of deoxyadenosine in the plasma of the SCID:ADA$^-$ child suggests possible complete clearance of deoxyadenosine by the kidney. Adenosine levels in the affected cells (lymphocytes, Table 2) of the SCID:ADA$^-$ patient are not as markedly-elevated as are the ribonucleotide pools in the same cells (22). However, the amount of adenosine found in lymphocytes is elevated in comparison to plasma, erythrocytes, and urine. The source of adenine in these cells and other tissues in humans remains an enigma.

This study was primarily directed toward development of an analytical method for measuring adenosine in biological tissues. As indicated by the different limits of detection for various tissues, application of this method to other adenine derivatives will probably require prior clean-procedures or other means of component separation to achieve maximal sensitivity. These problems arise due to the excessive amounts of nucleotides and other unidentified fluorescent materials present. However, such problems are probably not insurmountable and modifications of the methodology reported here should enhance the possibility of measuring other adenine components.

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1. The abbreviations used are: ADO, adenosine; dADO, deoxyadenosine; ADE, adenine; TUB, tubercidin; cAMP, cyclic adenosine-3',5'-monophosphate; SCID, severe combined immune deficiency disease; and ADA, adenosine deaminase.

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