ANALYSES OF ADENOSINE AND ADENINE NUCLEOTIDES IN BIOLOGICAL MATERIALS BY FLUORESCENCE REACTION–HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A previous method of determination of adenine compounds by high-performance liquid chromatography, using bromoacetaldehyde as a fluorescent reagent and a column of Hitachi gel No. 3012-N, was improved and extended to biological materials, especially to measure enzyme activities. A column packed with finer beads, Hitachi gel No. 3013-N, was found to be better than that of No. 3012-N, judging from the analysis time and resolution. ADP, from the hydrolysis of ATP by Na, K-ATPase, was determined quantitatively, and the enzyme activity was inhibited with ouabain. cAMP obtained from ATP by reaction with adenylate cyclase was also determined in the presence of various concentrations of L-epinephrine or sodium fluoride. The ATP levels in human blood were determined, and the cellular levels of ATP and ADP in neuroblastoma NIE 115 were examined as a function of cell growth.

INTRODUCTION

Kochetkov *et al.*[1] found a fluorescence reaction of chloroacetaldehyde with adenine bases. The fluorescent derivatives were characterized by Secrist *et al.*[2]. Yoshioka and Tamura[3] established a method of determination of the derivatives by high-performance liquid chromatography (HPLC). Kuttesch *et al.*[4] reported a clinical application of HPLC and found an abnormal excretion of deoxyadenosine in patients with immunodeficiency diseases.

Yoshioka *et al.*[5] reported that adenine compounds such as adenosine, AMP, cAMP, ADP and ATP were converted with a new fluorescent reagent, bromoacet-

aldehyde, into fluorescent derivatives (1,N^6-ethenoadenine derivatives), which are well separated on a column of macroreticular anion-exchange resin, Hitachi gel No. 3012-N (No. 2). In this study, the method was improved by using finer beads, Hitachi gel No. 3013-N (No. 3), and extended to measure the compounds in biological materials and to determine enzyme activities.

EXPERIMENTAL

Materials

Bromoacetaldehyde was prepared and crystallized according to the method of Schukovskaya *et al.*[6]. Na,K-activated ouabain-sensitive ATPase from dog kidney, and alumina (neutral), were obtained from Sigma (St. Louis, MO, U.S.A.) and M. Woelm (F.R.G.), respectively. The other chemicals, of reagent grade, were obtained commercially.

HPLC

The fluorescence reaction and HPLC were performed according to the previous method[5]. The No. 3 (mean diameter: 5 μm) and No. 2 (7 μm) resins were packed into tubes of the same size (12.5 cm × 4.6 mm) at 130 kg/cm² in the same manner. The column was maintained at 45°C. The mobile phase consisted of 0.025 M citric acid/0.4 M sodium chloride/0.05 M disodium hydrogenphosphate buffer (pH 5.0)–methanol (1:1, v/v). The flow-rate was 0.3 ml/min, maintained with a Twinkle pump (Jasco, Tokyo, Japan) or a Familic 300 pump (Jasco) connected to an Autosampler AS L350 (Jasco). The eluate was monitored by a fluorescence spectrophotometer FP-110 (Jasco)[5].

Assay of Na,K-ATPase inhibition with ouabain

The reaction mixture contained 200 mM Tes [N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid]–Tris [Tris(hydroxymethyl)aminomethane] (pH 7.4), 500 mM sodium chloride, 25 mM potassium chloride, 25 mM magnesium sulphate, 5 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid] and 200 mg/l of Na,K-activated ouabain-sensitive ATPase. To 20 μl of the latter solution were added 40 μl of a sample and the mixture was preincubated at 37°C for 20 min. The enzyme reaction was initiated by adding 40 μl of 5 mM ATP at 37°C. After incubation for 20 min, the reaction was stopped by boiling the incubate for 10 min. To 25 μl of the mixture were added 25 μl of 0.2 M phosphate buffer (pH 7.0) and 5 μl of 1.9 M bromoacetaldehyde. The reactions of ATP and ADP must be performed at pH 7, since their pyrophosphate linkages are hydrolyzed to some extent at low pH as described[5]. The fluorescence reaction was carried out as described previously[5].

Ouabain solutions (40 μl) of various concentrations were added to 20 μl of the reaction mixture. The inhibitory activity was calculated by

Percentage of inhibitory activity $= (X_0 - X_i)/(X_0 - X_{100}) \cdot 100$

where X is the peak-height ratio of ADP/(ADP + ATP) in the chromatogram, X_{100} is X at 10^{-3} M ouabain, which inhibited the ATPase completely, X_0 is X without ouabain and X_i is X at given concentrations of ouabain or other effectors.

Assay of adenylate cyclase activity

Fat cells were isolated from epididymal adipose tissue of Wistar rats (150 g) by the method of Rodbell[7]. Then, fat cell ghosts, prepared according to the method of Birnbaumer *et al.*[8], were used as an enzyme preparation.

The ghosts were suspended in the assay medium, consisting of 3.0 mM ATP, 3.6 mM magnesium chloride, 7.3 mM theophylline, 0.36 mM glycylglycine, 0.18 mM sodium sulphate, and 0.1% bovine serum albumin in 30 mM phosphate buffer (pH 7.6) at 37°C. The enzyme reaction was initiated by the addition of 10 μl of L-epinephrine or sodium fluoride solution. After incubation for 20 min, the reaction was stopped by boiling the incubate for 30 s. The solution was applied to a column (15 mm × 5 mm) packed with alumina. The column was eluted with 150 μl of water. To 150 μl of eluate were added 50 μl of 1 M acetate buffer (pH 5.0) and 20 μl of 1.9 M bromoacetaldehyde. The fluorescence reaction was carried out as described previously[5]. A second HPLC step was performed with a column (50 cm × 2 mm) of Hitachi gel No. 3010 (Hitachi, Tokyo, Japan), a macroreticular polystyrene resin without ion-exchange groups, as described previously[3].

Analysis of adenine compounds in blood

Adenine compounds in human whole blood were determined as follows. Blood (1 ml), drawn from an human brachial vein, was added to 250 μl of 4 M perchloric acid, chilled on ice, and homogenized in a glass homogenizer. After removal of proteins by centrifugation at 6000 g for 20 min, 50 μl of the supernatant were added to 10 μl of 4 M potassium hydroxide and the neutralized mixture was centrifuged at 3500 g for 5 min. The adenine compounds in the supernatant were derivatized as described previously[5]. The adenine compounds in human plasma were determined as described previously[5].

Analysis of adenine compounds in neuroblastoma cells

Mouse neuroblastoma N1E 115 cells were kindly provided by Dr. T. Deguchi, Department of Medical Chemistry, Tokyo Metropolitan Institute for Neurosciences, and grown in Dulbecco's modified Eagle minimal essential medium, supplemented with 8% foetal calf serum, in an humidified atmosphere of 10% carbon dioxide and 90% air at 37°C. The cells were collected by centrifugation at 200 g for 4 min and suspended in phosphate-buffered saline (0.14 M sodium chloride/3 mM potassium chloride/0.01 M phosphate buffer, pH 7.4). Two procedures were used for the determination of adenine compounds. In one, to 400 μl of the suspension were added 100 μl of 4 M perchloric acid, chilled on ice. The cells were homogenized in a glass homogenizer and centrifuged at 6000 g for 20 min. After removal of potassium perchlorate by centrifugation, to 25 μl of the supernatant were added 25 μl of 0.2 M phosphate buffer (pH 7) and 5 μl of 1.9 M bromoacetaldehyde. The fluorescence reaction was carried out as described earlier.

In the second procedure, 20 μl of the aldehyde were added to 100 μl of the above-mentioned cell suspension, since the aldehyde was hydrophobic and was expected to penetrate into the cells, and the mixture was heated at 85°C for 20 min. After removal of proteins by centrifugation at 300 g for 20 min, 10 μl of the diluted supernatant were injected into the HPLC system.

RESULTS

The adenine compounds were better separated by isocratic elution on No. 3 than No. 2 resin under the same conditions, previously reported by Yoshioka *et al.*[5], as shown in Fig. 1. The columns of both these resins were stable for more than a year.
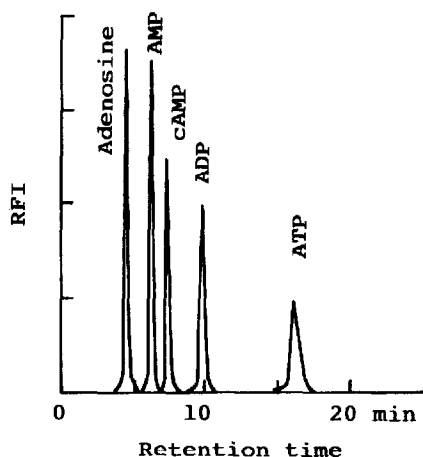

Fig. 1. Chromatogram of authentic adenine compounds on a column of resin No. 3. A solution (50 μl) of 1 μM adenine compounds in 0.1 M phosphate buffer (pH 7.0) was added to 5 μl of 1.9 M bromoacet-aldehyde, and then the mixture was heated as described previously[5]. The reactant (10 μl) was injected into the HPLC system described. RFI = Relative fluorescence intensity of the peak.

For comparison of columns containing resins No. 3 and No. 2, the analysis time and resolution were examined using a standard adenine mixture of adenosine, AMP, cAMP, ADP and ATP, after reaction with bromoacetaldehyde. As summarized in Table I, the analysis time on No. 3 was about half of that on No. 2 and the resolution afforded by No. 3 was higher than that by No. 2. The theoretical plate numbers of adenosine (3200), AMP (3700), cAMP (5300), ADP (3600) and ATP (3600) on No. 3 were larger than those on No. 2.

TABLE I

RETENTION TIME AND RESOLUTION OF ADENINE COMPOUNDS ON COLUMNS OF RESINS NOS. 3 AND 2

Ado = Adenosine.

Resin	Retention time (min)					Resolution			
	Ado	AMP	cAMP	ADP	ATP	Ado — AMP	— cAMP	— ADP	— ATP
No. 3	5.3	6.9	7.9	10.2	16.1	2.3	1.2	2.8	6.1
No. 2	5.3	8.4	10.1	15.1	31.4	1.7	1.0	1.3	2.5

The capacity factors of AMP and cAMP gradually decreased from 0.6 to 0.2, and 0.8 to 0.2, respectively as the final concentration of sodium chloride in the mobile phase increased from 0.1 to 0.3 M. The capacity factor of polyanionic ADP decreased from 3.1 to 0.2, as the sodium chloride concentration increased from 0.1 to 0.3 M. The one of ATP was changed from 12.1 to 0.6. Under the same experimental conditions, the separations of adenosine from AMP, cAMP from ADP, and ADP from ATP became smaller, whereas that of cAMP and AMP was constant, except in the case of 0.1 M sodium chloride. In the experiment with 0.3 M sodium chloride, ATP and ADP were co-eluted. Judging from these results, 0.2 M sodium chloride was optimal as shown in Fig. 1.

As the ratio of the buffer and methanol in the mobile phase was changed from 7:3 to 4:6 (v/v), the separations of adenosine from AMP, cAMP from ADP and ADP from ATP increased. The separation of cAMP from AMP decreased at the lower ratio but they were still completely separated at 60% methanol. The capacity factors of ADP and ATP increased as a function of the methanol concentration, but those of cAMP and AMP were not affected. This suggested that the separation of adenine compounds on this resin could be affected not only by ion exchange but also by other mechanisms, such as hydrophobic adsorption on the poly(styrene–divinyl). A ratio of 5:5 was appropriate for reducing the analysis time as shown in Fig. 1.

The deoxyadenine compounds were also well separated on resin No. 3 with a mobile phase consisting of 0.025 M citric acid/0.3 M sodium chloride/0.05 M disodium hydrogenphosphate buffer (pH 5.0)–methanol (3:2, v/v). The retention times of deoxyadenosine, deoxy-AMP, deoxy-cAMP, deoxy-ADP and deoxy-ATP at a flow-rate of 500 μl/min were 3.1, 4.1, 4.8, 7.0 and 14.2 min, respectively.

Na,K-ATPase was inhibited by ouabain, which blocks the interaction of potassium ions with the potassium sites. Josephson and Cantley[9] reported that 50 μM of ouabain inhibited over 98% of the activity of the Na,K-ATPase from dog kidney. In the presence of ATPase and an excess of ouabain, the highest peak of ATP appeared concomitantly with smaller amounts of ADP and AMP, spontaneously degradated or hydrolyzed by non-specific phosphatases. In the absence of ouabain, the peak of ADP increased, whereas the peak of ATP decreased. A typical sigmoidal dose–response curve was obtained, over the range from 10^{-9} to 10^{-3} M ouabain, and an approximately linear relationship was observed between the concentrations of 10^{-7} to 10^{-5} M.

cAMP was generated from ATP by adenylate cyclase from fat cell ghosts in the presence of L-epinephrine. In the adsorption chromatography with Hitachi gel No. 3010, cAMP was eluted at 30 min, followed by adenosine and adenine, and AMP, ADP and ATP were eluted at the void volume. Activation curves were drawn for 10^{-7}–10^{-3} M of L-epinephrine or 10^{-3}–10^{-2} M of sodium fluoride, respectively. The sigmoidal curves were in fair agreement with the data from a tracer experiment with a radioisotope, reported by Birnbaumer and Rodbell[10].

The adenine compounds in an human whole blood were determined, as shown in Fig. 2. The concentrations of ATP and ADP were 700 and 100 μM, respectively. In Fig. 3 the ATP and ADP concentrations in a plasma in the presence of 1 mg EDTA in 1 ml of blood were 3.2 and 2.4 μM, respectively. The time course of the ATP level in an human plasma in the presence of 25 mg EDTA in 1 ml of blood was examined by two methods. In one, at 6-min intervals during 30 min, the blood was
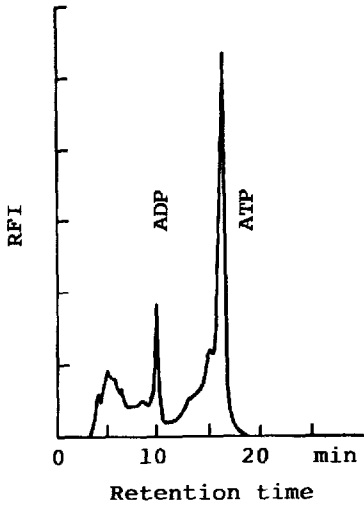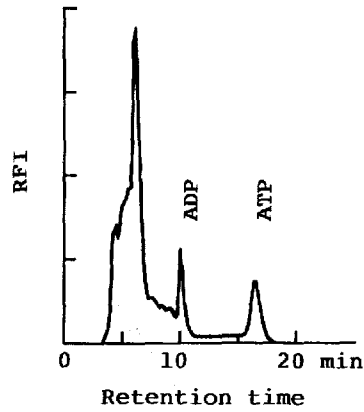
Fig. 2. Chromatogram of human whole blood.

Fig. 3. Chromatogram of human plasma.

centrifuged and the ATP in plasma was determined. The amount of ATP (7 μM) in the plasma decreased as a function of time and became non-detectable after 30 min. The half-life of ATP in the plasma of the stored blood at 4°C was less than 4 min. In the second method, ATP in plasma, which was separated immediately after the blood was drawn, did not change significantly during 1 h.

The cellular levels of ATP and ADP in neuroblastoma N1E 115 were measured by two different methods. When the cells were allowed to react directly with bromoacetaldehyde, which penetrated into the cells, two high peaks appeared and were followed by relatively low peaks due to ADP and ATP. The amounts of ADP and ATP were 31 ± 1.6 (S.D.) and 14 ± 0.56 pmol/μg of protein ($n = 4$), respectively. In the second method, the adenine compounds in the supernatant of the cells were determined as shown in Fig. 4. The amounts of ADP and ATP were 11 ± 0.47 and
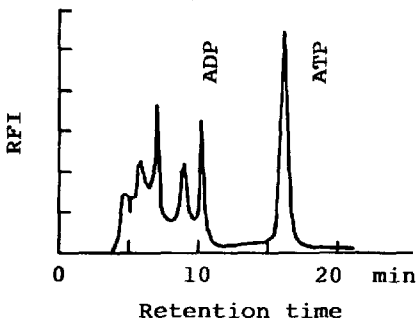
Fig. 4. Chromatogram of neuroblastoma N1E 115 cells, homogenized and allowed to react with bromoacetaldehyde.
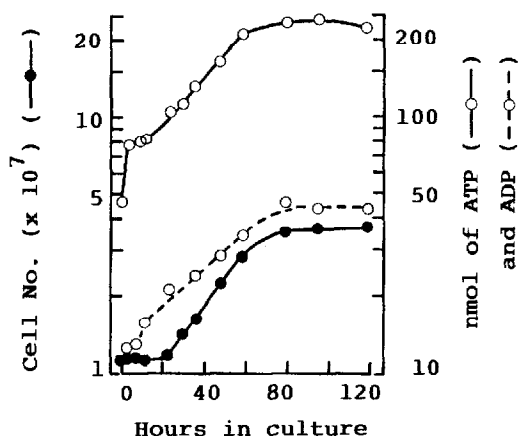
Fig. 5. Cellular levels of ATP and ADP in neuroblastoma N1E 115 cells during growth.

51 ± 1.8 pmol/µg of protein ($n = 4$), respectively. The changes in the ATP and ADP levels in neuroblastoma N1E 115 during cell growth were examined by the latter method. A typical cell growth curve was obtained by plotting the total cell numbers against time in culture, as shown in Fig. 5. Cellular levels of ATP and ADP increased in step with cell growth changes. The recoveries of AMP, ADP and ATP, which were added with 4 M perchloric acid, were 93 ± 4.2, 93 ± 6.6 and 99% ± 3.3 ($n = 4$) respectively from neuroblastoma cells.

DISCUSSION

So far, the determination of adenine compounds by HPLC has been performed with regular-sized columns, packed with either ion-exchange resins[11,12] or reversed-phase resins[13,14]. In almost all the methods, they were eluted by inconvenient gradient methods in order to shorten the analysis times.

Judging from the reduction in analysis time and enhancement of resolution, we have improved our previous determination of adenine compounds, which was performed on resin No. 2, by introducing gel No. 3.

Recent investigations have demonstrated some advantages of the use of microbore columns. A semi-microbore column (25 cm × 1.5 mm) packed with No. 3 was tested for the determination of adenine compounds. Although, under optimum conditions, the analysis time with the semi-microbore was longer than that with the regular (12.5 cm × 4.6 mm) column, the resolution and theoretical plate numbers were worse. Thus, the semi-microbore column packed with the anion-exchange resin was not as effective for the determination of adenine compounds as our HPLC system.

There have been earlier methods for measuring the activities of Na,K-ATPase and adenylate cyclase. The former was measured by a radiochemical[15], Fiske–Subbarow[16] or luciferin-luciferase[17] method. The latter was determined by a radiochemical method or radioimmunoassay (RIA)[18], which required either a specific antibody or a binding protein. Our method is more specific and at least 1000 times higher in

sensitivity than that of Fiske–Subbarow. The enzyme method with luciferase has a high sensitivity, but is plagued with a high background and it is unable to measure ATP and ADP simultaneously. Our HPLC method has a sensitivity comparable to RIA, because the threshold of the detection via fluorescence is as low as 1 pmol, without requiring the use of radioisotopes. RIA is sometimes subject to interference from salts, proteins and tissue culture medium[19], whereas these factors do not interfere in the fluorescence reaction of adenine compounds with bromoacetaldehyde. Additionally, the appearance of unexpected peaks in the chromatogram can reveal contamination(s) of enzyme(s) in an enzyme preparation.

Shimada *et al.*[20] reported an assay procedure for Na,K-ATPase from guinea pig hearts by HPLC with an UV monitor. Our results were similar to theirs but our method is more sensitive, specific and suitable for small samples. Recently, a circulating inhibitor of Na,K-ATPase[21], which was assumed to be an digitalis-like factor[22], was found to be increased in essential hypertension. We are now examining the inhibitory effects of the factor from human urine on Na,K-ATPase with our method. Details will be reported elsewhere.

Hartwick *et al.*[23] reported that plasma contained 86 nucleosides, bases and other low-molecular-weight UV-absorbing materials. Fibroblasts[24] and erythrocytes[25] also contain many UV-absorbing materials. Peak identification is very critical. The chromatograms of blood (Figs. 2 and 3) and neuroblastoma N1E 115 (Fig. 4) showed several peaks which were easily identifiable, because the reaction of bromoacetaldehyde is relatively specific for adenine bases. Although cytosine and guanine compounds also react with bromoacetaldehyde, the relative fluorescence intensities of cytosine products are negligible and those of guanines are less than one-hundredth of those of adenine derivatives in the fluorescence detector. As the concentrations of guanines and cytosines in biological materials, except RNA and DNA, are less than one-tenth to one-hundredth of those of adenines, they are practically indetectable in the chromatograms.

The sample preparation prior to HPLC is very critical in assessing the actual nucleotide content, as pointed out by Hartwick and Brown[12]. Recently, Born and Kratzer[26] reported that the concentration of ATP in human blood was about 2 μM, 2–4 s after vascular injury, and increased to 20 μM 3–5 min after injury. In our experiments, the ATP concentration in human plasma varied from 1 to 10 μM, probably due to the physiological conditions. The half-life of ATP in stored blood is a few minutes in the presence of EDTA. To measure ATP levels in plasma accurately, blood cells should be collected and separated as soon as possible, even in the presence of EDTA, to prevent ATP in plasma from being taken up and hydrolyzed by blood cells and ecto-ATPases of erythrocytes and leukocytes[27], respectively. The shoulder on the ATP peak in Fig. 2 is assumed to be due to deoxy-ATP, which was subtracted from the peak of ATP. The complete separation of deoxy-ATP was easily done by decreasing the sodium chloride in the mobile phase. This method will be described elsewhere. The energy charge[28] of neuroblastoma N1E 115 was calculated from

$$(ATP + 1/2\ ADP)/(ATP + ADP + AMP)$$

The value (0.86) from Fig. 4 was higher than that (0.35) from the direct reaction of the cells with bromoacetaldehyde, indicating that the homogenization of cells in the

procedure for the determination of adenine nucleotides is better than in the latter procedure.

ACKNOWLEDGEMENTS

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