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Direct injection of blood samples into a high-performance liquid chromatographic adenine analyser to measure adenine, adenosine and the adenine nucleotides with fluorescence detection

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ABSTRACT

Adenine (Ade), adenosine (Ado) and its nucleotides such as AMP, cAMP, ADP and ATP in blood or plasma were determined by a high-performance liquid chromatographic (HPLC) adenine analyser with fluorescence detection. In order to inject samples directly into the HPLC system without pretreatment except dilution, the analyser consisted of two systems each, having three columns (pre-, mini- and analytical). A precolumn with an inlet filter of pore size 40 μm was common to both systems and packed with Butyl-Toyopearl 650-M to remove hydrophobic compounds and blood cell membranes. In the system for analysis of the nucleotides, a mini-column of Hitachi anion-exchange gel 3013-N was used for adsorbing AMP, cAMP, ADP and ATP. The adsorbed nucleotides were separated by the Hitachi gel 3013-N analytical column. In the other system for analysis of Ado and Ade, they were adsorbed on a Develosil ODS-5 mini-column and separated by an Asahipak GS-320H size-exclusion analytical column. The adenine compounds in each eluate were derivatized on-line in a 15-m reaction coil at 115°C with bromoacetaldehyde as the fluorescent reagent in each mobile phase for the analytical column, and detected by spectrofluorimetry.

ATP, ADP and AMP were accurately determined by the direct injection of hamster, rat and human whole blood. Authentic Ade and Ado were well separated and Ado in human plasma was determined, but it was difficult to determine it in rat plasma owing to interference from an unknown compound.

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INTRODUCTION

ATP, ADP, AMP, cAMP, adenosine (Ado) and adenine (Ade) are involved in a series of metabolic pathways in biological systems, not only producing carriers of high energy but also playing important roles as chemical mediators[1].

Yoshioka and co-workers[2–5] reported that adenine compounds were converted with bromoacetaldehyde as a fluorescent reagent into fluorescent derivatives, which were systematically determined by high-performance liquid chromatography (HPLC) with fluorescence detection. In one system described by Yoshioka and co-workers[2,4,5], adenine compounds from biological samples such as blood and cultured cells were extracted with perchloric acid and then prederivatized with the aldehyde. In the other system[3], the extracted compounds were separated and derivatized on-line with a prototype analyser. In these analyses, pretreatment of the samples was essential in order to maintain the efficiency of the columns. The conditions for pretreatment were critical to prevent the degradation of adenine nucleotides.

In this study, we tried to develop methods for the direct injection of biological samples, including cells, into the prototype analyser without any pretreatment such as deproteinization, except sample dilution. The method consisted of two systems, one for analyses of anionic compounds such as ATP, ADP, cAMP and AMP, and the other for analyses of the neutral compounds such as Ado and Ade.

EXPERIMENTAL

Materials

Bromoacetaldehyde was prepared and crystallized according to the method of Schukovskaya *et al.*[6]. Butyl-Toyopearl 650-M (BT 650-M) (44-88 μm) was purchased from Tosoh (Tokyo, Japan). Hitachi gel 3013-N (3 μm), consisting of particles of macroreticular anion-exchange resin that had been sieved from the original size of mean diameter 5 μm, was kindly provided by Hitachi (Tokyo, Japan). Develosil ODS-5 (ODS-5) (5 μm) was kindly provided by Mr. M. Nomura (Nomura Chemical, Aichi, Japan). Asahipak GS-320H for size exclusion was donated by Asahi Chemical Industry (Kanagawa, Japan).

All adenine compounds were dissolved in 0.1 *M* phosphate buffer (pH 7.0) to make 0.5–10 μM standard solutions and stored at -80°C until HPLC analysis. β-Nicotinamide adenine dinucleotide (NAD), flavine adenine dinucleotide (FAD) and adenosine-5′-diphosphoribose (ADP-ribose), obtained from Sigma (St. Louis, MO, U.S.A.), were dissolved in 0.1 *M* phosphate buffer to make 10 μM solutions. Other chemicals were of analytical-reagent grade.

Columns

For the determination of the nucleotides, a common precolumn (3 cm × 4.6 mm I.D.) with an inlet filter of pore size 40 μm was packed with BT 650-M to remove cell debris and proteins, and a mini-column (1 cm × 4.0 mm I.D.) to adsorb only the nucleotides and an analytical column (5 cm × 4.6 mm I.D.) were packed with Hitachi gel 3013-N. For determination of Ade and Ado, a precolumn packed with BT 650-M was used, a mini-column (3 cm × 4.6 mm I.D.) to adsorb Ade and Ado was packed with ODS-5 and the analytical column (25 cm × 7.6 mm I.D.) was packed with

Asahipak GS-320H. The inlet and outlet filters of the columns, except the precolumn, were equipped with filters of pore size 2 μm.

Analyser system for determination of adenine nucleotides

A flow diagram of the analyser is shown in Fig. 1. The precolumn at ambient temperature was equilibrated with 10–20% (v/v) acetonitrile in water as eluent I and the mini-column and the analytical column were equilibrated with a mixed solution of 0.008–0.1 *M* bromoacetaldehyde (finally selected as 0.05 *M*), 0.15 *M* sodium chloride and 15% acetonitrile containing 0.025 *M* citrate buffer (pH 4.0) (eluent II). Just before the sample injection, the pre- and mini-columns were connected with each other. After a 10-μl sample injection, eluent I was passed through the precolumn into the mini-column at a flow-rate of 0.1–0.5 ml/min for 2–10 min (optimally 0.3 ml/min for 4 min) to adsorb the nucleotides on the mini-column. Then the mini-column was connected with the analytical column maintained at 45°C and eluted with eluent II at a flow-rate of 0.3 ml/min. The eluate was heated in a reaction coil of 3–18 m × 0.25 mm I.D. at 70–125°C (15 m at 115°C was finally selected) with an FIU Reaction Unit RU-150F (Jasco) which was filled with polyethylene glycol 400, and detected using a Model 820-FP spectrofluorimeter (Jasco). The wavelengths of excitation and emission were set at 254 and 400 nm, respectively. One division of relative fluorescence intensity (RFI) corresponded to 1.5 mV. The chart speed of the recorder was set at 0.5 cm/min.

Analyser system for determination of Ade and Ado

The flow diagram of the analyser is as shown in Fig. 1. The pre- and mini-columns at ambient temperature were equilibrated with 0–7% (v/v) acetonitrile as eluent I′. The analytical column maintained at 40°C was equilibrated with 0.05 *M* bromoacetaldehyde–0.15 *M* sodium chloride–15% acetonitrile containing 0.025 *M* citrate buffer (pH 5.0) (eluent II′). After a 10-μl sample injection, Ado and Ade were adsorbed on the mini-column by passing eluent I′ at a flow-rate of 0.3 ml/min for 2–6 min (finally selected as 4 min). After the adsorption, the mini-column was connected

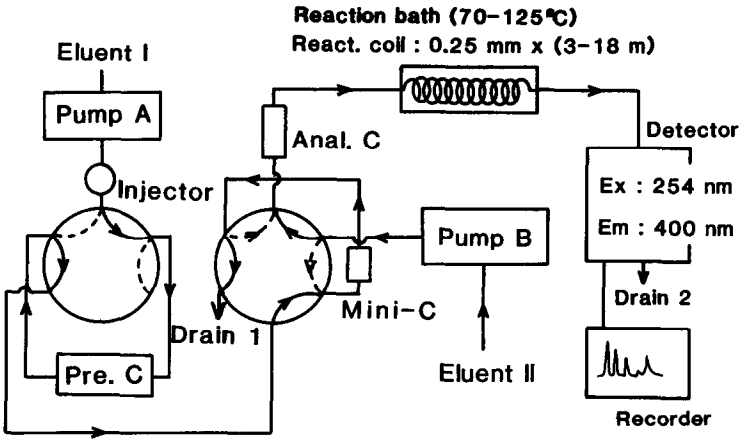


Fig. 1. Schematic diagram of the analyser.

with the analytical column and eluted with eluent II′ containing bromoacetaldehyde at a flow-rate of 0.5 ml/min. The eluate was heated and monitored as described above. The chart speed of the recorder was set at 0.3 cm/min.

Determination of adenine compounds in blood and plasma

Hamster or rat whole blood from a common carotid vein or human whole blood from a brachial vein was taken by using a 1-ml plastic disposable syringe rinsed with 1 ml of physiological saline (0.9%) containing heparin (100 U/ml). To measure systematically the nucleotides in the whole blood, 5 μl of the blood were diluted 10–100-fold with 0.32 *M* sucrose and 10 μl of the diluted solution (net injection volume of 0.1–1 μl of the blood) was injected into the analyser system.

Blood from a healthy human volunteer's (10 ml) was withdrawn from a brachial vein into a 50-ml plastic disposable syringe containing 10 ml of a mixed solution of 20 U heparin, 0.01% (w/v) dipyridamol and 10 m*M* manganese chloride. The blood was centrifuged at 600 *g* for 5 min at 4°C and the resulting supernatant plasma was stored at −80°C until use. To determine Ado and Ade, 10 μl of the plasma were injected into the analyser system.

RESULTS

The adenine nucleotides were well separated by the analyser system and eluted from the Hitachi gel 3013-N analytical column in the order AMP, cAMP, ADP and ATP, as shown in Fig. 2. The analytical time was *ca.* 26 min. Ade and Ado were not detected by the present analyser system despite injecting large amounts (100 nmol each) of Ade and Ado as they were not adsorbed on the mini-column. Hence the developed system was specific for determination of the adenine nucleotides, as expected.

The effect of the acetonitrile concentration in eluent I on the peak heights of

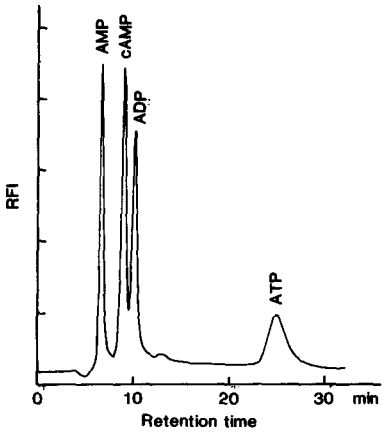
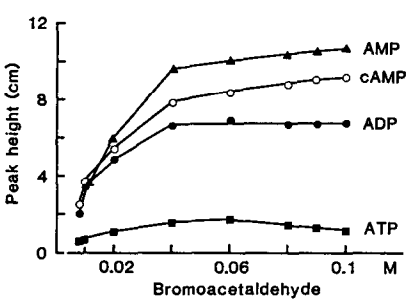


Fig. 2. Chromatogram of authentic adenine compounds obtained with the analyser for nucleotides. A 10-μl volume of 3 μ*M* adenine compounds in 0.1 *M* phosphate buffer (pH 7.0) was injected into the analyser system. The retention time means the time elapsed just after the connection of the mini- with the analytical column.

ATP, ADP and AMP was examined in the range 10–20%. As all the peaks at 15% acetonitrile were the heighest, this concentration was adopted. A flow-rate of eluent I of 0.3 ml/min gave the best results in the range 0.2–0.5 ml/min. The retention times of the nucleotides remained constant with variation in the flow-rate of eluent I, because their retention times were completely dependent on the flow-rate of eluent II for the analysis. The peak height of AMP was constant during 4 min of adsorption, but decreased as a function of adsorbing time between 4 and 10 min, whereas the peak heights of cAMP, ADP and ATP still remained constant after 10 min. Calibration graphs for AMP, cAMP, ADP and ATP were linear from 0.5 to 100 pmol injected. By direct injection of 10 μl of rat whole blood diluted 20-fold with 0.32 *M* sucrose, the highest peaks of AMP, ADP and ATP at 4-min adsorption were also obtained under the optimum conditions established. Judging from these results, 15% acetonitrile in the eluent I, a flow-rate of 0.3 ml/min and an adsorption time of 4 min were selected as optimum for the pre- and mini-columns.

Derivatization of the nucleotides eluted from the analytical column was examined. The peak heights of the nucleotides almost reached plateaux at *ca.* 0.05 *M* bromoacetaldehyde in eluent II when the 10-m reaction coil was used at 120°C, as shown in Fig. 3. Fig. 4 shows the effect of the length of the reaction coil on the analysis. Judging from the heights of peaks and the analytical time, 15 m was selected as the optimum length. The effect of the temperature of the reaction coil on the derivatization is shown in Fig. 5. The peak heights of cAMP, ADP and ATP, but not AMP, were maximum at a heating temperature of 110°C in the range 70–125°C examined, without changing their retention times. Therefore, 115°C was selected for the subsequent experiments.

The adenine nucleotides in hamster whole blood were determined under the optimum conditions developed above, as shown in Fig. 6. From the calibration graphs for the authentic adenine compounds, the concentrations of ATP, ADP and AMP in the whole blood were calculated to be 813, 129 and 8 μmol/l, respectively. The unknown peaks 1 and 2 were assumed to be adenine-related compounds, because



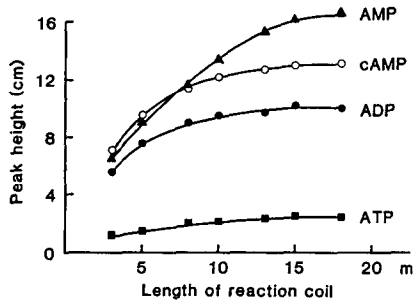
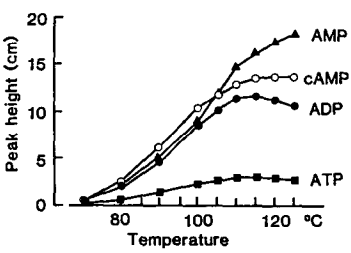


Fig. 3. Effect of bromoacetaldehyde concentration on peak heights. A 40-pmol amount of the adenines was injected into the analyser system. Eluent I was passed through the precolumn into the mini-column at a flow-rate of 0.3 ml/min for 4 min. The eluate was heated in a 10-m reaction coil at 120°C. Data points: ▲ = AMP; ○ = cAMP; ● = ADP; ■ = ATP.

Fig. 4. Effect of length of reaction coil on peak heights. A 40-pmol amount of the adenines was injected and eluted with eluent II containing 0.05 *M* bromoacetaldehyde. The other condition and data points were the same as in Fig. 3, except the length of the coil.



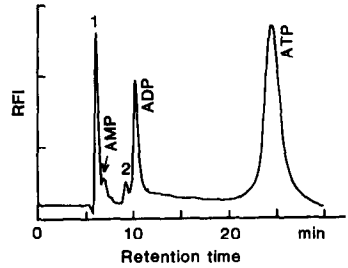


Fig. 5. Effect of heating temperature on peak heights. The length of the reaction coil was 15 m and the other conditions were the same as in Fig. 4, except the heating temperature. Data points as in Fig. 3.

Fig. 6. Chromatogram of hamster whole blood. Peaks 1 and 2 are unknown compounds.

the fluorescent reaction and detection were selective for adenine bases. As the retention times of NAD, FAD and ADP-ribose and also cAMP corresponded to the unknown peak 2, cAMP was difficult to determine. The nucleotides in hamster whole blood were also determined at 35-min intervals during 140-min storage (Table I). The total concentration of AMP, ADP and ATP and the concentration of ATP decreased to 69 and 63% of the original concentrations, respectively. However, the concentration of AMP and the value of the "energy charge" (EC) were constant during 140 min of storage. Table II gives the concentrations of ATP and ADP in whole blood determined with the present analyser system and with the previously described prederivatization method[2,4,5]. The values obtained with the present system were reasonable.

In the other system, Ado and Ade were well separated by the analyser system and the analytical time was *ca.* 30 min, as shown in Fig. 7. Ado and Ade were adsorbed on the mini-column packed with ODS-5 using 0–5% acetonitrile as eluent I' but the amounts adsorbed were markedly decreased when 6 and 7% acetonitrile were used. When the acetonitrile content in eluent I' was lower than 2%, ATP, ADP and

TABLE I

CHANGES IN LEVELS OF ADENINE NUCLEOTIDES IN HAMSTER WHOLE BLOOD DURING STORAGE

The hamster whole blood was diluted 20-fold with 0.32 *M* sucrose and stored at 4°C. This set of experiments was performed three times with similar results.

Time (min)	*Concentration in whole blood (μmol/l)*			*Total adenines*[a] *(μmol/l)*	*EC*[b]
	ATP	*ADP*	*AMP*		
0	8	129	812	949	0.92
35	10	192	717	919	0.88
70	11	217	671	899	0.87
105	12	192	545	749	0.86
140	10	137	512	659	0.88

[a] AMP + ADP + ATP.

[b] EC (energy charge) is calculated from the concentrations (ATP + 0.5 ADP)/(AMP + ADP + ATP).

TABLE II

COMPARISON OF LEVELS OF ATP AND ADP IN VARIOUS WHOLE BLOODS DETERMINED BY THE PRESENT AND PREDERIVATIZATION METHODS

As reported previously by Yoshioka and co-workers[2,4,5], adenine compounds in each whole blood were extracted with perchloric acid, prederivatized with bromoacetaldehyde and analysed by HPLC. The values represent means ± S.D. of three and five experiments using the present and the prederivatization methods, respectively.

Method	*Concentration in whole blood (μmol/l)*					
	Hamster		*Rat*		*Human*	
	ATP	*ADP*	*ATP*	*ADP*	*ATP*	*ADP*
Present	810±33	130±4.9	600±27	115±5.3	750±35	110±5.3
Prederivatization	780±32	140±6.0	580±26	120±6.0	700±32	100±5.0

ATP were also adsorbed on ODS-5. Hence a 5% acetonitrile content was optimum for selective adsorption.

When the 1 cm × 4.6 mm I.D. column was used as the mini-column, the pH of eluent I′ containing 5% acetonitrile was a critical factor for the adsorption of Ado and Ade. At pH 4, ATP, ADP and AMP and also Ado and Ade were adsorbed on the column, but only Ado and Ade were selectively adsorbed at pH 7.4. Judging from these results, 5% acetonitrile in 2 m*M* phosphate buffer (pH 7.4) was selected as optimum for eluent I′. The peak height of Ado reached a plateau from 3 to 6 min

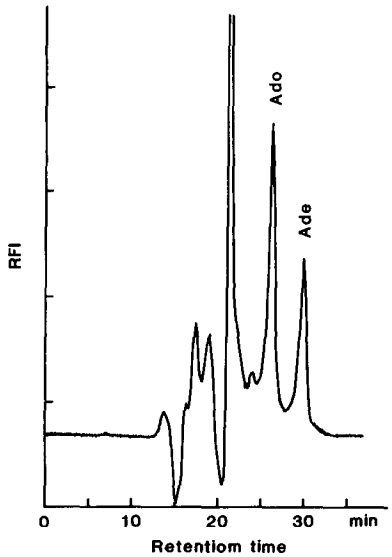


Fig. 7. Chromatogram of authentic adenine compounds obtained with the analyser for Ado and Ade. A 30-pmol amount of the adenines was injected into the analyser system. Eluent I, 5% acetonitrile in 2 m*M* phosphate buffer (pH 7.4), was passed through the precolumn into the mini-column at a flow-rate of 0.3 ml/min for 4 min. The other conditions were the same as optimized with the analyser for the nucleotides.

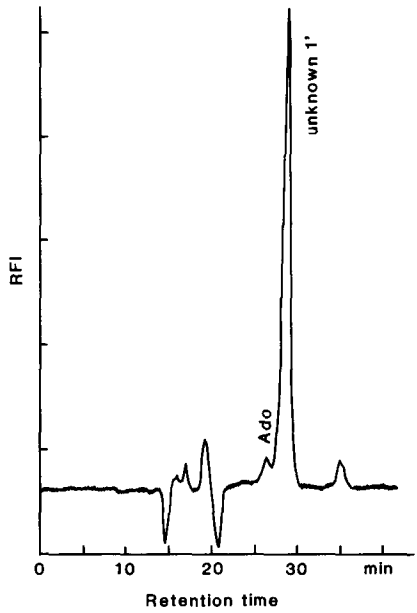


Fig. 8 Chromatogram of human plasma.

adsorption after the sample injection. On the other hand, the peak of Ade after 4 min of adsorption was the highest in the period 2–6-min. As both the pre- and mini-columns should be well equilibrated with eluent I′, timing of the column switching from connection of the mini- with the analytical column to connection of the pre- with the mini-column was a critical factor for the successive determination of Ado and Ade. As the peak heights of Ado and Ade were nearly same from 3 to 9 min after the connection of the mini- with the analytical column, the timing of the column switching was selected to be 5 min. The calibration graphs were linear over the range 5–50 pmol of Ade and Ado injected.

Under the conditions established above, Ado and Ade in healthy human plasma were determined and the results are shown in Fig. 8. The concentration of Ado was *ca.* 0.3 μmol/l and no Ade could be detected. It was difficult to determine the content of Ado in rat plasma by the large intereference from the unknown peak 1′, which was also observed in human plasma. However, NAD, FAD and ADP-ribose passed through the mini-column, and did not appear in the chromatogram. Hence the system was specific for the determination of Ado and Ade.

DISCUSSION

Tamai *et al.*[7–9] established individual methods for the determination of hydrophobic, moderately hydrophobic and hydrophilic drugs in whole blood by direct injection of the blood into HPLC systems. For the determination of hydrophilic drugs, they introduced BT 650-M in a precolumn equipped with an inlet filter of pore size 40 μm, allowing blood cells to pass into the column and cell debris, cell mem-

branes and proteins to be trapped in the column. The adsorbed materials such as proteins, lipids and cell debris on the BT 650-M precolumn were removed by washing with 0.5% sodium dodecylsulphate and then methanol after the direct injection of 200 μl of whole blood into the HPLC system[9].

Adenine compounds are relatively hydrophilic, and the introduction of BT 650-M as the precolumn was adequate for development of the present system. In this study, 0.1–1 μl of the whole blood was sufficient to determine the contents of adenine compounds based on the high sensitivity of the fluorescence detection and the selectivity of derivatization with bromoacetaldehyde. Cytosine and guanine compounds also react with bromoacetaldehyde, but the relative fluorescence intensities of their products are 100 times lower than those of adenine derivatives and they are therefore, virtually not detectable in the chromatograms. The yield of the fluorescent derivatization in the reaction coil was not calculated, but the peak heights of the nucleotides reached plateaux at 115°C, as shown in Fig. 5, suggesting that the yield is high enough for quantitative determination. Judging from the reproducibility of the peak heights of each nucleotide, the system was stable for at least 100 injections without regeneration of the pre- and mini-columns. In our system, polymerized bromoacetaldehyde and the adsorbed compounds such as lipids and proteins are washed out after every 50 sample injections with 50% methanol.

As pointed out by Hartwick and Brown[10], sample preparation in HPLC is very critical when determining the actual nucleotide contents. ATP and ADP are easily metabolized during sample preparation. The concentrations of ATP and ADP in the dilute human blood analysed with the present system were 750 and 110 μM, respectively, which are close to those obtained by the prederivatization method described previously[4,5]. The ATP concentration in rat whole blood obtained by our methods (Table II) was higher than the 521 μmol/l obtained with UV detection[11]. By direct injection of hamster whole blood into the analyser system, a high EC value (0.92) was obtained. Hence the analyser for the nucleotides is useful for assessing the EC values of various cells or clinical samples.

Ado is a metabolic product of ATP and a mediator of coronary vasodilatation[12], hormonal secretion[13], cyclic nucleotide formation[14–16], neurotransmission[17] and immune response[18,19]. Several methods for measuring Ado with enzymatic spectrophotometric[20], radiochemical[21], fluorimetric[22], enzymatic isotope dilution[23], radioligand binding[24] and radioimmunological[25] techniques have been reported. HPLC has been used for the determination of Ado and Ade in plasma[26,27]. As the Ado content in plasma is very low, an ODS column such as Sep-Pak C_{18} has commonly been used to concentrate Ado from plasma, but elution of Ado from the Sep-Pak C_{18} and evaporation of the eluate were time consuming. Boos *et al.*[28] developed effective column-switching techniques for the direct injection of biological fluids such as human serum, urine and breast milk into a high-performance affinity chromatograph using phenylboronic acid bonded precolumn material. Ado was clearly separated from the other ribonucleosides by the automated on-line analysis of ribonucleosides with UV detection. However, a large amount of serum (500 μl) was required. From the clinical point of view, the system should be set up for small amounts of biological samples.

In this study, the Ado concentration in normal human plasma was found to be about 0.3 μmol/l, which corresponds closely with the levels of 0.29[25], 0.51[26], 0.35[28] and 0.28 μmol/l[29] reported previously.

In our system, an unexpected appearance of an unknown peak which intereferes with the determination of Ado was found with the plasma samples, particularly rat plasma. When the plasma and blood cells were separated immediately by centrifugation, the unknown compound was detected in the chromatogram of the plasma whereas no such peak could be detected in that of the blood cells, indicating that the unknown compound exists mainly in the plasma. The unknown compound was stable against the endogenous adenosine deaminase. Deoxyadenosine corresponded to the retention time of the unknown peak 1′ but was completely degradaded in the rat plasma (data not shown). Characterization of the compound is being undertaken and will be described elsewhere. Using our analyser system, Ado in crude hepatic mitochondria from guinea pig was accurately determined (data not shown).

The analyser for Ado and Ade was stable for at least *ca.* 15 sample determinations without washing the BT 650-M and ODS-5 columns with a higher concentration of the acetonitrile or other washing solutions.

For the simultaneous determination of Ade and Ado with the nucleotides, a new packing material for the mini-column that will adsorb Ado and Ade in the presence of bromoacetaldehyde would be required. Further, development of a microinjection method for 0.01–0.1-μl volumes would also be required for the real direct injection of biological samples into the analyser.

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REFERENCES

1 J. L. Gordon, *Biochem. J.*, 233 (1986) 309.
2 M. Yoshioka, K. Nishidate, H. Iizuka, A. Nakamura, M. M. El-Merzabani, Z. Tamura and T. Miyazaki, *J. Chromatogr.*, 309 (1984) 63.
3 M. Yoshioka, Z. Tamura, M. Senda and T. Miyazaki, *J. Chromatogr.*, 344 (1985) 345.
4 M. Yoshioka, K. Yamada, M. M. Abu-Zeid, H. Fujimori, A. Fuke, K. Hirai, A. Goto, M. Ishii, T. Sugimoto and H. Parvez, *J. Chromatogr.*, 400 (1987) 133.
5 M. Yoshioka, K. Yamada, M. M. Abu-Zeid, H. Fujimori, A. Fuke, K. Hirai, A. Goto, M. Ishii, T. Sugimoto and H. Parvez, *Supercritical Fluid Chromatography and Micro-HPLC*, VSP, Utrecht, 1989, p. 181.
6 L. L. Schukovskaya, S. N. Ushakov and N. K. Galania, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1962) 1692.
7 G. Tamai, H. Yoshida and H. Imai, *J. Chromatogr.*, 423 (1987) 163.
8 G. Tamai, H. Yoshida and H. Imai, *J. Chromatogr.*, 423 (1987) 147.
9 G. Tamai, H. Yoshida and H. Imai, *J. Chromatogr.*, 423 (1987) 155.
10 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651.
11 K. Shimizu, S. Kawazoe, M. Shiraishi and T. Asahi, *Yakugaku Zasshi*, 103 (1983) 1323.
12 R. M. Berne, *Am. J. Physiol.*, 204 (1963) 317.
13 J. Wolff and G. H. Cook, *J. Biol. Chem.*, 252 (1977) 687.
14 H. Shimuzu, C. R. Creveling and J. Daly, *Proc. Natl. Acad. Sci. U.S.A.*, 65 (1970) 1033.
15 C. Londos and J. Wolff, *Proc. Natl. Acad. Sci. U.S.A.*, 74 (1977) 5482.
16 M. Saito, *Biochim. Biophys. Acta*, 498 (1977) 316.
17 J. Daly, *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides*, Raven Press, New York, 1979, p. 229.

18 E. R. Giblett, J. E. Anderson, F. Cohen, B. Pollara and H. J. Meuwissen, *Lancet*, 2 (1972) 1067.
19 G. C. Mills, F. C. Schmalstieg, K. B. Trimmer, A. S. Goldman and R. M. Goldblum, *Proc. Natl. Acad. Sci. U.S.A.*, 73 (1976) 2867.
20 H. M. Kalckar, *J. Biol. Chem.*, 167 (1947) 429.
21 E. Randerath, C.-T. Yu and K. Randerath, *Anal. Biochem.*, 48 (1972) 172.
22 G. Avigad and S. Damle, *Anal. Biochem.*, 50 (1972) 321.
23 D. H. Namm and J. P. Leader, *Anal. Biochem.*, 58 (1974) 511.
24 P. A. Olsson, C. J. Davis, M. K. Gentry and R. B. Vomacka, *Anal. Biochem.*, 85 (1978) 132.
25 T. Sato, A. Kuninaka, H. Yoshino and M. Ui, *Anal. Biochem.*, 121 (1982) 409.
26 M. C. Capogrossi, M. R. Holdiness and Z. H. Israili, *J. Chromatogr.*, 227 (1982) 168.
27 E. K. Jackson and A. Ohnishi, *Hypertension*, 10 (1987) 189.
28 K.-S. Boos, B. Wilmers, E. Schlimme and R. Sauerbrey, *J. Chromatogr.*, 456 (1988) 93.
29 J. Ontyd and J. Schrader, *J. Chromatogr.*, 307 (1984) 404.