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Note

Analyser of adenine nucleotides*

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All organisms contain a variety of adenine nucleotides. ATP, ADP and AMP are involved in the energy transformations, and cAMP from ATP plays an important role as a second messenger in the transmission of hormone and nerve information between cells. These adenine nucleotides undergo rapid enzymic transformation into each other. Concentrations of the adenine nucleotides are much higher than those of the other purine and pyrimidine nucleotides. It is desirable to determine the adenine nucleotides simultaneously.

High-performance liquid chromatography (HPLC) is used for the systematic analysis of water-soluble compounds. Many HPLC methods have been developed for the adenine nucleotides, with detection based on the UV absorption of the adenine base. However, they are not selective or sensitive enough to measure the adenine nucleotides in biological materials (e.g. ref. 1).

In 1976, Yoshioka and Tamura introduced a new method, in which the adenine nucleotides were treated with chloroacetaldehyde, and the fluorescent 1-N^6-ethenoadenine (ε-adenine) nucleotides produced were separated by HPLC

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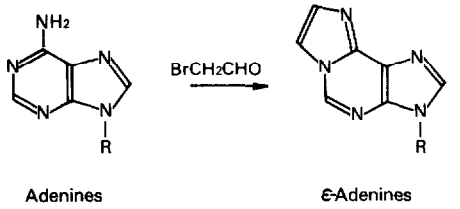

Fig. 1. Fluorescent reaction of adenines.

[2]. This method was improved in three ways: (1) the fluorescent reagent bromoacetaldehyde was used (Fig. 1); (2) a porous polystyrene anion-exchange resin enabled isocratic elution; and (3) the use of a fluorescence spectrophotometer meant that micro-HPLC could be performed [3]. The method was applied to measure adenosine in synaptosomes from guinea-pig cerebral cortex [4] and adenine nucleotides released with catecholamines from adrenal chromaffin cells [5].

In this study, the fluorescent reaction was coupled on-line with HPLC to make a specific analyser. The fluorescent reagent was passed through a column in an eluent, and the fluorescent reaction was carried out in a heating coil after the separation.

EXPERIMENTAL

Materials

Bromoacetaldehyde was prepared and crystallized according to the method of Schukovskaya et al. [6]. The other chemicals, of reagent grade, were commercially obtained. All the adenine nucleotides of 0.5—10 μM each were dissolved in 0.1 *M* phosphate buffer (pH 7.0), used as standard solutions and stored at − 80°C until HPLC analysis.

Fluorescent reaction for pre-column derivatization of adenine nucleotides

The standard solution was thawed at 40°C and 100 μl of the solution were added to 10 μl of 1.9 *M* bromoacetaldehyde. According to the previous method [3], the mixed solution was heated in a Reacti-Vial (Pierce, Rockford, IL, U.S.A.) at 80°C for 15 min and stored at 4°C for HPLC analysis.

Analyser for adenine nucleotides

The analyser was constructed as shown in Fig. 2. The eluent consisted of 0.025 *M* citric acid—0.05 *M* disodium hydrogen phosphate—0.4 *M* sodium chloride (pH 5.0) and acetonitrile (4:1, v/v). Bromoacetaldehyde was added to the eluent in a final concentration of 0.1 *M*. The flow-rate was 0.1 ml/min from a Familic 300S pump (Jasco). A 35 × 4.6 mm I.D. column of Hitachi gel No. 3012-N (porous polystyrene polymer beads for anion exchange, mean diameter 7 μm, Hitachi, Tokyo, Japan) was maintained at 45°C with an inlet pressure of 5 kg/cm^2. It was connected to a reaction coil (30 m × 0.1 mm I.D.). The coil was heated at 100°C with a water-bath, and the exit was connected to a fluorescence spectrophotometer FP-110 (Jasco) described in the previous paper [3]. In the spectrophotometer, light of wavelength 253.7 nm from a low-

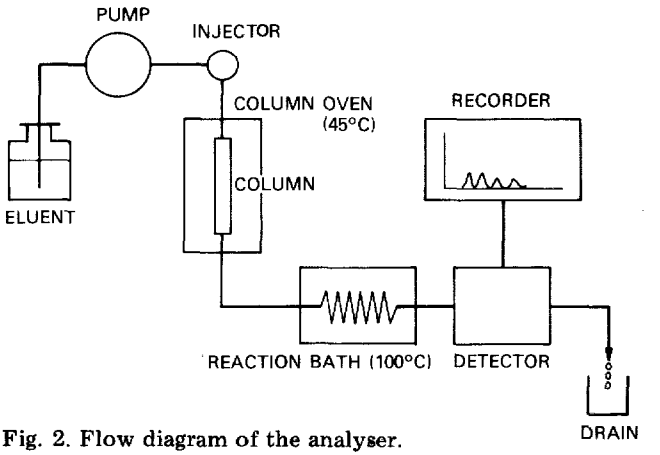

Fig. 2. Flow diagram of the analyser.

pressure mercury lamp was dispersed by an excitation concave grating and focused on a 3-μl flow-through cell. The fluorescence at 400 nm was collected by an emission concave grating.

RESULTS

The idea for the analyser arose from the fact that the pH for the separation (pH 5) was the same as the optimum pH for the fluorescent reaction described in our previous paper [3]. The fluorescent reagent is neutral, inert during the separation and reactive with the adenine nucleotides when heated after the column separation.

The flow system was finally designed as shown in Fig. 2. To save reagent, we chose a small column and examined the composition of the eluent. Acetonitrile does not react with bromoacetaldehyde and was used in place of methanol [3]. The ε-adenine nucleotides were best separated with the eluent containing 20% (v/v) acetonitrile, judging from the capacity factors as shown in Fig. 3.

The chromatogram obtained under these conditions is shown in Fig. 4A. The ε-adenine nucleotides (1 pmol each) were clearly separated with the eluent without bromoacetaldehyde. When the eluent contained 0.1 *M* bromoacetaldehyde, the peak heights of ε-adenines decreased, probably owing to absorption of the excitation light by polymerized bromoacetaldehyde (Fig. 4B). The adenine nucleotides were separated with the eluent containing 0.1 *M* bromoacetaldehyde and derivatized at 100°C (Fig. 4C), and had retention times shorter than those of the ε-adenines. The peaks of the adenine nucleotides did not appear when the coil was not heated above 20°C (Fig. 4D). This indicates that the reaction is very slow at 20°C and pH 5.0 in the mobile phase used.

Fig. 5 shows that the peak height reaches a plateau at ca. 100°C.

The calibration curves for the adenine nucleotides were linear from 0.5 to 10 pmol injected, and the coefficient of variation of the peak height ratios of AMP, cAMP and ADP to ATP were 0.6, 1.3 and 1.2% (intra-assay, n=6) and 0.9, 1.5 and 1.2% (inter-assay, n=10), respectively.

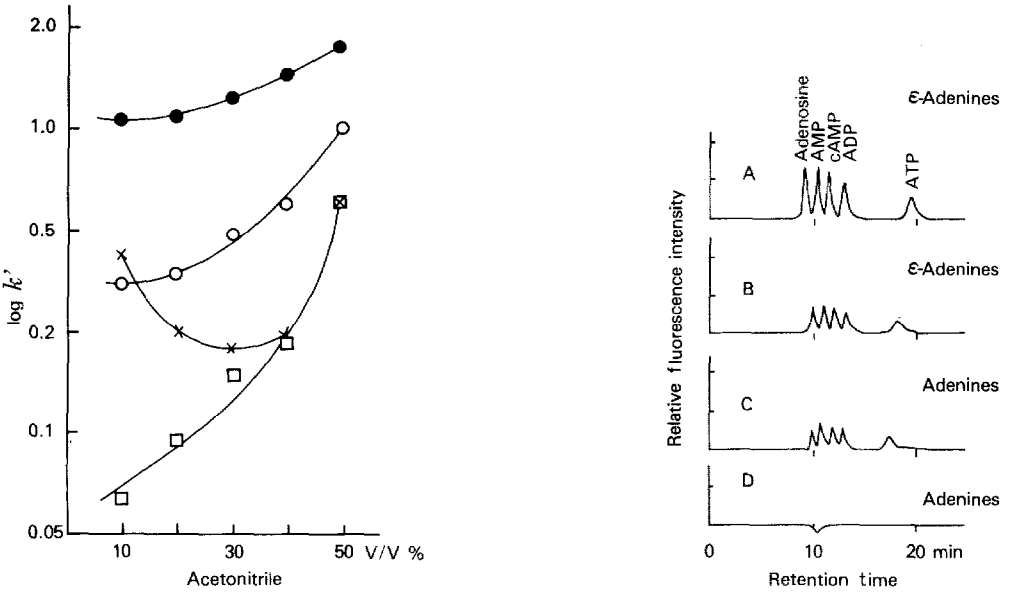

Fig. 3. Effect of acetonitrile concentration in the eluent on capacity factor. ε-Adenosine is considered to be eluted not retained. The capacity factor (k') is defined as $k' = (t_R - t_0)/t_0$, where t_0 is the retention time of adenosine and t_R is the retention time of the other compounds. Data points: □ = ε-AMP; × = ε-cAMP; ○ = ε-ADP; ● = ε-ATP. The buffer (pH 5.0) was mixed with various concentrations of acetonitrile. The other conditions were the same as in Fig. 4A.

Fig. 4. Chromatograms of adenines treated with bromoacetaldehyde in pre-column and post-column reactions. The adenines (1 pmol each) reacted as described in the text were injected. The ε-adenines were separated (A) without and (B) with 0.1 *M* bromoacetaldehyde through the reaction coil at 100° C as described in the text. The adenines (1 pmol each) were separated with the eluent with 0.1 *M* bromoacetaldehyde through the reaction coil at 100° C (C) and 20° C (D).

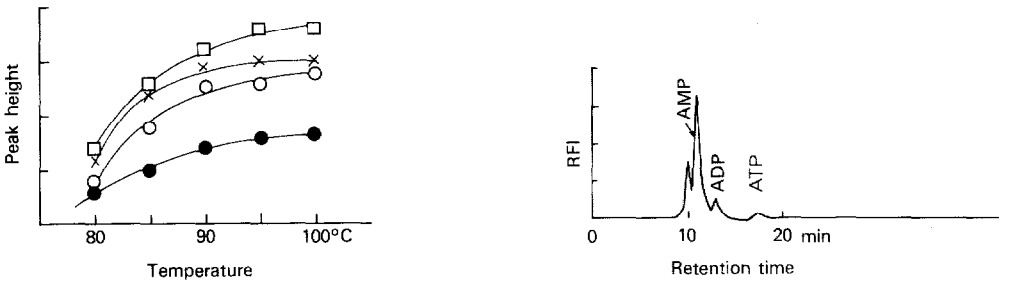

Fig. 5. Effect of the temperature of the reaction coil. The adenines (3 pmol each) were separated with the eluent with 0.1 *M* bromoacetaldehyde through the reaction coil at various temperatures. Data points as in Fig. 3.

Fig. 6. Chromatogram of brain extract. A fresh brain of a rat (male Wistar, 210 g) was homogenized in 3 ml of 0.4 *M* perchloric acid and centrifuged at 15 000 *g* for 30 min. A portion of the supernatant was diluted 50-fold with the eluent, and 1 µl of the diluted solution was injected.

The column of the analyser was stable for more than one year. The sensitivity increased if the flow-through cell was enlarged: however, the background also increased.

The pyrophosphate linkage in ADP and ATP is unstable to heat at low pH, so the post-column reaction has merit in this respect. In the ATP chromatogram such degradation did not occur, since ADP and AMP were not found.

The analyser was applied to measure the adenine nucleotides in a rat brain (Fig. 6). The deproteinized solution was diluted with perchloric acid and injected into the analyser. The amounts of AMP, ADP and ATP were $4.5 \cdot 10^{-7}$, $1.1 \cdot 10^{-7}$ and $0.68 \cdot 10^{-7}$ mol/g wet weight, respectively; these values are compatible with those described in the previous report [3].

DISCUSSION

The simple analyser for the adenine nucleotides described here involved a new way of mixing the fluorescent reagent, which was added to the eluent beforehand.

Hitherto, coloring reagents or fluorescent reagents have been mixed to give either a pre-column or a post-column reaction in on-line analysers [7, 8]. In all these systems, at least two pumps are required in order to adjust the pH, ensure proper mixing of the reagent, and maintain constant flow-rates. The last is essential for sharp resolution and high sensitivity.

In the present method, although a post-column reaction is carried out, a single pump is sufficient to elute and mix the adenine nucleotides separated with the fluorescent reagent. Hence pump problems occurred less often than with other systems.

The analyser has another particular characteristic. In our previous method [3], more than 10 μl of the sample solution had to be used for the quantitative handling of the fluorescent derivatization. In the present method, just 1 μl of the sample was injected into the analyser without the derivatization, making the procedure suitable for the minute amounts available from biological subjects.

Nor is it necessary to protect the unstable linkages of the adenine nucleotides. Degradation was avoided by decreasing the reaction temperature and using the buffer at neutral pH, where the concentration of the fluorescent reagent must be increased to obtain the higher yield in the previous method [3]. This analyser will be suitable for unknown samples containing such linkages.

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REFERENCES

1 R. Marquetant and R.S. Goody, J. Chromatogr., 280 (1983) 386.
2 M. Yoshioka and Z. Tamura, J. Chromatogr., 123 (1976) 220.
3 M. Yoshioka, K. Nishidate, H. Iizuka, A. Nakamura, M. Mahmoud, M.M. El-Merzabani, Z. Tamura and T. Miyazaki, J. Chromatogr., 309 (1984) 63.

4 K. Kobayashi, Y. Kuroda and M. Yoshioka, J. Neurochem., 36 (1981) 86.
5 Y. Kuroda, M. Yoshioka, K. Kumakura, K. Kobayashi and T. Nakajima, Proc. Jap. Acad., 56B (1980) 660.
6 L.L. Schukovskaya, S.N. Ushakov and N.K. Galania, Izvest. Akad. Nauk SSSR, Otd. Khim. Nauk, (1962) 1692.
7 M.S.F. Ross, J. Chromatogr., 141 (1977) 107.
8 J.F. Lawrence and R.W. Frei, Chemical Derivatization in Liquid Chromatography, Elsevier, Amsterdam, 1976.