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**Note**

# Fluorimetric determination of adenine and adenosine and its nucleotides by high-performance liquid chromatography

MASANORI YOSHIOKA and ZENZO TAMURA
*Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113 (Japan)*
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In a study on adenyl cyclase, the determination of micro-amounts of adenine and related compounds was required. Recently, high-performance liquid chromatographic (HPLC) methods were applied in order to separate nucleic acid components[1]. Their sensitivity and specificity however, are not sufficient to permit the determination of these adenine-based compounds. Secrist and co-workers[2,3] found that the 1,$N^6$-etheno derivatives of adenosine and its nucleotides, obtained by reaction with chloroacetoaldehyde, were strongly fluorescent. Avigad and Damle[4] applied this reaction to the determination of total adenine contents at concentrations as low as 10 pmoles.

We attempted to separate the fluorophores derived from adenine and adenosine and its nucleotides by HPLC and to determine them quantitatively at pmole levels, the results obtained being described below.

## EXPERIMENTAL

### *Materials*

Adenine, adenosine, adenosine 5′-phosphate (AMP) and chloroacetoaldehyde were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Adenosine 3′,5′-cyclic monophosphate (cAMP) was kindly supplied by Seishin Pharmaceutical Co. (Chiba, Japan) and adenosine 5′-triphosphate (ATP) by Takeda Chemical Industries (Osaka, Japan). 1,$N^6$-Etheno derivatives of adenine, adenosine, AMP, cAMP and ATP were synthesized according to the method of Secrist *et al.*[2] and Barrio *et al.*[3]. The purity of these derivatives was checked by thin-layer chromatography[2] and elemental analysis.

### *Methods*

A solution (500 $\mu$l) containing various amounts of adenine and related compounds was placed together with 100 $\mu$l of 1 *M* acetate buffer (pH 4.5) and 10 $\mu$l of 4 *M* chloroacetoaldehyde in a glass-stoppered test tube. The tube was stoppered and heated in a boiling water bath for various periods of time. After cooling it, 5 $\mu$l of the solution were injected into a high-performance liquid chromatograph.

The chromatograph used was as follows. A glass tube (500 × 2 mm I.D.) was packed with Hitachi gel No. 3010, particle diameter *ca.* 25 $\mu$m (Hitachi, Tokyo Japan) and maintained at 40°. The mobile phase consisted of 0.1 *M* phosphate buffer

(pH 7.0)–methanol (3:2). The flow-rate was set at 0.1 ml/min by a Type KWU-32H double plunger micropump kindly supplied by Kyowa Seimitsu Co. (Tokyo, Japan). The column inlet pressure was 5 kg/cm$^2$. The eluate was monitored by a FLD-1 fluorescence detector (Shimadzu, Kyoto, Japan) equipped with a low-pressure mercury lamp of maximum energy at 253.7 nm, a Shimadzu EX-2 primary filter that transmitted radiation in the range 250–400 nm, a quartz flow-through cell of 10-$\mu$l capacity and a Shimadzu EM-3 secondary filter that transmitted radiation of wavelengths above 410 nm.

## RESULTS

Authentic 1,N$^6$-etheno derivatives of adenine, adenosine, AMP and cAMP were well separated under the prescribed HPLC conditions, as shown in Fig. 1, although AMP and ATP derivatives were not. Calibration curves for peak heights *versus* amounts injected with a 5-$\mu$l microsyringe were linear up to several tens of pmoles. These peak heights were reproducible and the coefficient of variation was less than 0.8% with 16 pmoles of cAMP.

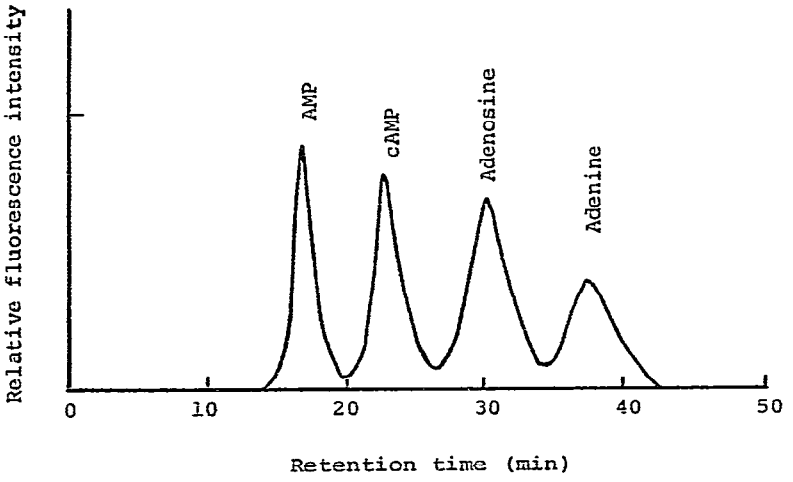


Fig. 1. Chromatogram of 4 pmoles of authentic 1,N$^6$-etheno derivatives.

Chloroacetoaldehyde was allowed to react with a mixture of adenine, adenosine, AMP and cAMP as described under Experimental. The chromatographic profile of the reaction mixture was essentially similar to that of the authentic materials shown in Fig. 1, except that a peak due to a product of the reaction of chloroacetoaldehyde with itself overlapped that of AMP (Fig. 2). The reagent blank was low and constant, and was subtracted so as to obtain the true value for AMP. The reaction of chloroacetoaldehyde with cAMP reached a plateau within 30 min (Fig. 3). The yield, calculated by reference to the calibration curves, was 80%, and was constant for the various concentrations of the four compounds, the working curves for the determination being linear, as shown in Fig. 4. The minimum amount that could be detected was 1 pmole. The intensity of the fluorescences did not change during 1 week, as shown in Fig. 5.

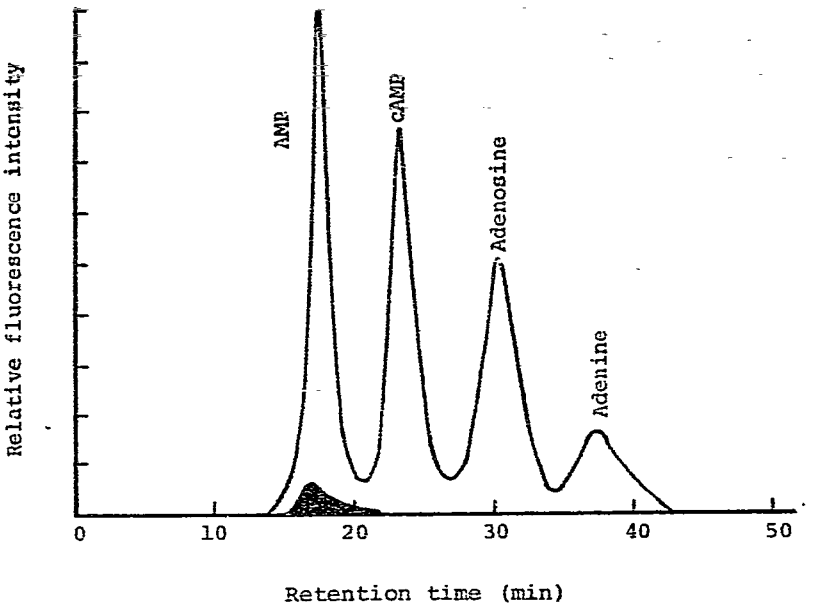


Fig. 2. Chromatogram of reaction mixture. A solution containing adenine, adenosine, AMP and cAMP, each at $4 \times 10^{-6}$ *M* concentration, was made to react with chloroacetoaldehyde for 30 min as described in the text; 5 $\mu$l of the mixture were injected. The black area shows the reagent blank.

## DISCUSSION

Adenine and related compounds at pmole levels are separated and determined by the proposed method. The solid phase, Hitachi gel No. 3010, is a porous polystyrene resin and does not need to be regenerated, in which respect it differs from the ion-exchange resins usually used for the separation of nucleic acid components[1]. The fluorimetric detector and fluorophores are very stable, and continuous analysis can

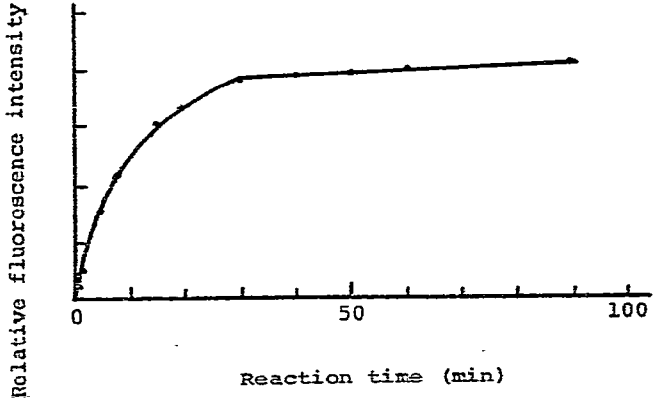


Fig. 3. Time of the reaction of chloroacetoaldehyde with cAMP. A $4 \times 10^{-6}$ *M* solution of cAMP was treated as described in the text; 5 $\mu$l of the reaction mixture were injected into the high-performance liquid chromatograph.

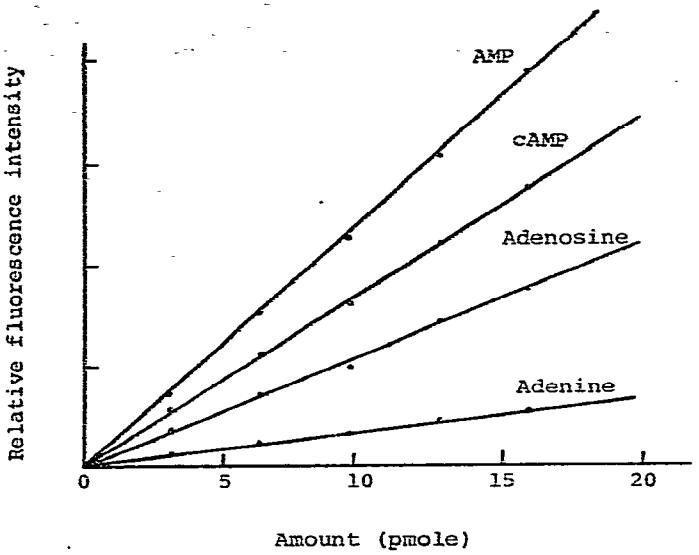


Fig. 4. Working curves for adenine and related compounds. Solutions of the mixture at $0.8 \times 10^{-6}$–$4 \times 10^{-6}$ *M* concentration were made to react for 30 min as described in the text and 5 $\mu$l of each were injected.

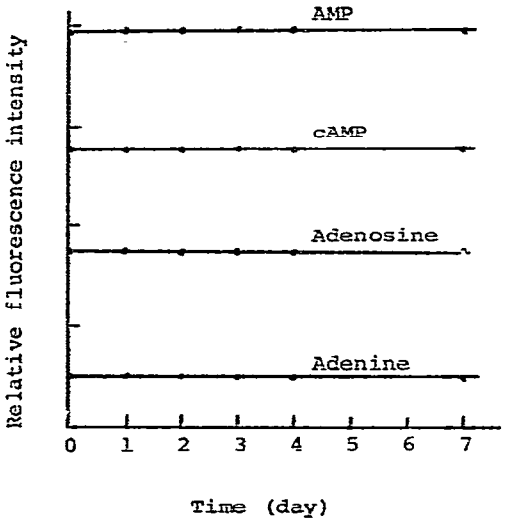


Fig. 5. Stability of fluorescence of the 1,N$^6$-etheno derivatives. The reaction mixture of Fig. 2 was stored at 4° and 5 $\mu$l were injected.

therefore be carried out. Polyanionic compounds such as AMP and ATP emerge in the same position and must be resolved by use of ion-exchange resins[1].

Among other nucleic bases only cytosine produces a weak fluorescence at the different wavelengths[2,3], which, however, is cut off by the secondary filter, thus making the present method specific for adenine and related compounds.

The present method is conventional and gives promise of wide application to

adenine-based compounds, compared with the radioimmunoassay[5] or the protein binding assay[6] of cAMP, which requires the use of highly specific binding proteins.

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