Journal of Chromatography, 309 (1984) 63—71
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2113

SENSITIVE FLUORIMETRY OF ADENINE-CONTAINING COMPOUNDS WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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(First received October 31st, 1983; revised manuscript received February 8th, 1984)

SUMMARY

A definitive method to determine adenine compounds simultaneously was established by introducing a new fluorescent reagent into high-performance liquid chromatography. Bromoacetaldehyde was the best reagent among the haloacetaldehydes examined. A quantitative reaction was obtained even for unstable ADP and ATP. A high resolution of adenine nucleotides was obtained using a column of Hitachi gel No. 3012-N. The method was applied to the measurement of cyclic AMP in urine, and ADP and ATP in brain and blood. Further, the sensitivity of the method was increased by a new fluorescence spectrophotometer constructed for micro-HPLC. Femtomole amounts of the adenine nucleotides were clearly separated.

*Presented at the 8th Symposium on Nucleic Acids Chemistry, Sapporo, August 21—23, 1980 (see ref. 18).

0378-4347/84/$03.00

INTRODUCTION

There are a number of adenine-containing compounds such as adenine nucleosides and nucleotides in natural and synthetic compounds, and development of a highly sensitive and selective method of determination is fundamental to progress in medical, biochemical and chemical studies.

Specific radioimmunoassay (RIA), enzymatic and protein binding assays for adenine compounds such as cyclic AMP (cAMP) [1] and adenosine [2] have been developed, if appropriate binding proteins are available.

Adenine compounds are not suitable for conversion into volatile derivatives for gas chromatography. A liquid-chromatographic method is useful for their systematic analysis. Recently, high-performance liquid chromatography (HPLC) has been applied to determine adenine compounds. Detection, however, is based on ultraviolet absorption of the adenine base and is not selective [3—6].

It is relevant to use a fluorescence reaction specific for the adenine base. Kochetkov et al. [7] found that chloroacetaldehyde reacted with adenine base to produce a 1-N^6-ethenoadenine derivative. Secrist et al. [8] examined the fluorescence characteristics of the derivatives.

Previously, we developed a method in which adenine compounds were quantitatively converted with chloroacetaldehyde into their etheno derivatives, which were separated by HPLC [9]. Kuttesch et al. [10] modified the method to determine adenosine and deoxyadenosine in patients with immunodeficiency diseases. Preston [11] also extended the method to separate adenine and related nucleotides in an extract of phytoplankton.

In this paper, we shall describe in detail the improved method which was communicated in brief [18]. A new fluorescence reagent, separation system and fluorescence detector were adopted. The method was applied to determine adenine compounds in biological samples.

EXPERIMENTAL

Materials

Bromoacetaldehyde was prepared and crystallized according to the method of Schukovskaya et al. [12]. Iodoacetaldehyde was prepared as described by Glinsky [13]. The other chemicals, of reagent grade, were commercially obtained.

Fluorescence reactions of haloacetaldehydes with adenine compounds

To 500 μl of 10 μM cAMP or a mixture of the adenine compounds (10 μM each) were added 200 μl of buffer (1 M citrate buffer pH 3, 1 M acetate buffer pH 3.5—5.5, or 1 M phosphate buffer pH 6.0—7.0) and 40 μl of 1.2 M bromoacetaldehyde in a Reacti-Vial (Pierce, Rockford, IL, U.S.A.). The vial was tightly closed and the mixed solution was heated at various temperatures (80°C or 100°C) for various times (0—40 min). In place of bromoacetaldehyde, 25 μl of 8 M iodoacetaldehyde dissolved in dioxane warmed at 60°C, or 10 μl of 4 M chloroacetaldehyde, were added. The reacted solution was analysed by HPLC to compare the reactivities of the haloacetaldehydes.

Quantitative reaction of the adenine compounds with bromoacetaldehyde

To 100 μl of the solution of adenine compounds in 0.1 *M* phosphate buffer (pH 7) or 1 *M* acetate buffer (pH 5) at various concentrations were added 10 μl (for pH 7) or 5 μl (for pH 5) of 1.9 *M* bromoacetaldehyde. The Reacti-Vial containing the mixed solution was heated at 80° C for 15 min and stored at 4° C until HPLC analysis.

High-performance liquid chromatography

A column (10 cm × 4.6 mm) of Hitachi gel No. 3012-N (porous polystyrene polymer beads for anion exchange, mean diameter of 7 μm, Hitachi, Tokyo, Japan) was maintained at 45° C. An eluent consisted of 0.025 *M* citric acid—0.05 *M* disodium hydrogen phosphate—0.4 *M* sodium chloride buffer (pH 5.0) and methanol (1:1, v/v). The flow-rate of the eluent was set at 0.2 ml/min with a Twincle pump (Jasco). The column inlet pressure was 10 kg/cm^2. The column was connected to a fluorescence detector FLD-1 (Shimadzu, Kyoto, Japan) equippped 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-μl capacity and an EM3 secondary filter that transmitted radiation of wavelengths above 400 nm.

Micro-HPLC

A PTFE tube (130 mm × 0.5 mm) was packed with Hitachi gel No. 3012-N and maintained at 12° C The flow-rate of the eluent mentioned above was set at 4 μl/min by a microsyringe-type pump Familic 100 N (Jasco).

The eluent was monitored by a fluorescence spectrophotometer.

Fluorescence spectrophotometer for micro-HPLC

The spectrometer was newly constructed and later named FP-110 (Jasco) (Fig. 1). The light from a low-pressure mercury lamp is filtered to cut off visible emissions, and is then introduced into an excitation monochromator through an entrance slit. The light beam is dispersed by an excitation concave grating (EXCG) of *F* 2.5 and focused on a flow-through cell of 1.5-μl capacity through an exit slit. The fluorescence is collected by the emission concave grating (EMCG), dispersed and focused on an exit slit of an emission monochromator. The emission photomultiplier (EMPM) converts the fluorescence

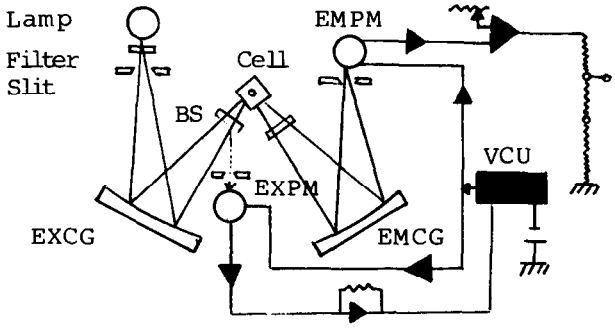

Fig. 1. A fluorescence spectrophotometer for micro-HPLC. For explanation of abbreviations, see text.

intensity to electrical signal. The fluctuation of the emission from the light source is compensated by monitoring a portion of the emission, which is split by the beam splitter (BS). The excitation signal level was maintained constant with the excitation photomultiplier (EXPM) for which high-tension voltage was regulated with a voltage control unit (VCU).

Determination of cAMP in human urine

Urine samples from healthy men were collected between 15.00 and 17.00 hours. To 0.5 ml of each urine were added 5 μl of 1 m*M* 2-deoxy cAMP as internal standard and 0.5 ml of water. Then 200 μl of the diluted urine were poured on to a column (5 mm × 4.5 mm) of Dowex 50W-X4 (H^+). The column was washed with 800 μl of water. To 50 μl of effluent were added 50 μl of 1 *M* acetate buffer (pH 5) and 5 μl of 1.9 *M* bromoacetaldehyde. The fluorescence reaction was carried out as described under Quantitative reaction.

Determination of adenine nucleotides in brain

A brain weighing 1.6 g was taken from a Wistar rat (210 g body weight) into 3 ml of 0.4 *M* perchloric acid chilled on ice. The brain was homogenized in a glass homogenizer. The homogenate was centrifuged at 15,000 *g* for 20 min. To 100 μl of the supernatant was added an equal volume of 1 *M* potassium bicarbonate and the mixture was centrifuged at 3500 *g* for 5 min. To 50 μl of the supernatant were added an equal volume of 0.2 *M* phosphate buffer (pH 7) and 10 μl of 1.9 *M* bromoacetaldehyde. The fluorescence reaction was carried out as described above. A 1-μl aliquot of the reacted solution was injected into the chromatograph.

Determination of adenine nucleotides in blood

Blood from human brachial vein was drawn into a tube containing disodium EDTA (100 mg/ml of blood). The tube was centrifuged at 3500 *g* for 5 min. To 100 μl of the plasma were added 25 μl of 4 *M* perchloric acid and the mixture was centrifuged at 15,000 *g* for 20 min. A 100-μl volume of the supernatant was reacted as described under Determination of adenine nucleotides in brain.

RESULTS

The adenine nucleotides were well separated without the aid of gradient elution, as shown in Fig. 2. The column (10 cm × 4.6 mm) used in this experiment had a higher resolution than the one (50 cm × 2 mm) previously reported [18], although the other conditions were the same. The column of Hitachi gel No. 3012-N was very stable and maintained constant resolution after one year of use. The deoxyadenine nucleotides were separated from the corresponding adenine nucleotides and eluted just in front of them as indicated for deoxy cAMP in Fig. 2.

It was demonstrated that each adenine nucleotide was converted with any haloacetaldehyde into the corresponding 1-N^6-ethenoadenine nucleotide, which was eluted at the same retention time, as shown in Fig. 2.

In Fig. 3, the pH optima of the reactions with bromoacetaldehyde and iodoacetaldehyde were 5.0 and 4.0, respectively, whereas with chloroacetaldehyde

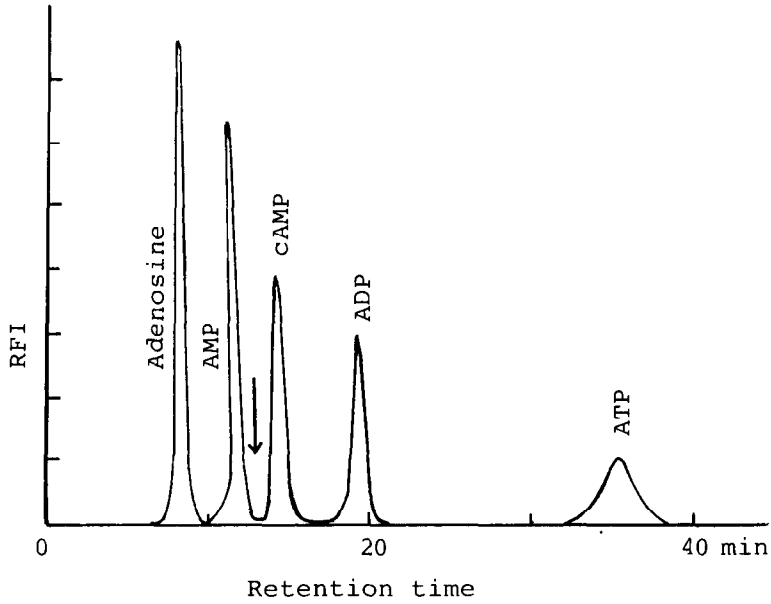

Fig. 2. Chromatogram of authentic compounds: 10 pmol of each were injected. Relative fluorescence intensity (RFI) of the peak was drawn. The arrow shows the location of the deoxy cAMP peak.

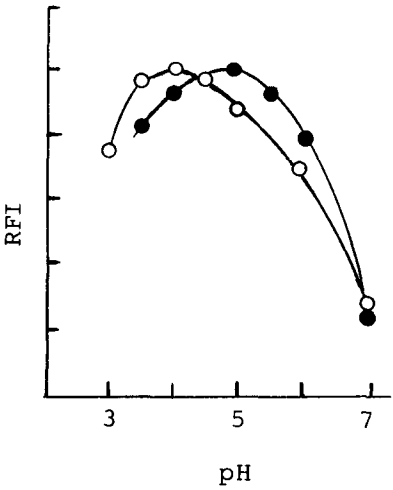

Fig. 3. Reactivities of haloacetaldehydes with cAMP at various pH values. •, Bromoacetaldehyde; ○, iodoacetaldehyde.

it was 4.5 as described before. The reactivity of bromoacetaldehyde was the strongest, as shown in Fig. 4. The reaction of bromoacetaldehyde reached a plateau at 100°C for 8 min or at 80°C for 15 min. Iodoacetaldehyde showed decreased fluorescence after the reaction maximum. Compared with standard synthesized 1-N^6-etheno cAMP, the yield of the reaction with bromoacetaldehyde at the plateau was 94%, while the reaction yield with chloroacetaldehyde was 80% at 100°C for 30 min. Thus, bromoacetaldehyde was adopted as the fluorescence reagent.

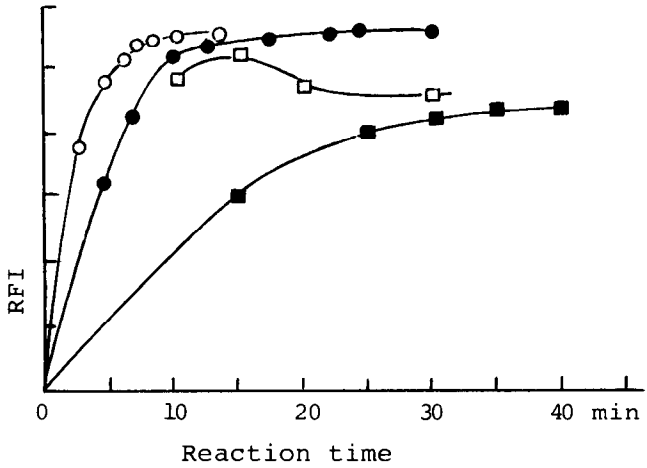

Fig. 4. Time courses of the reactions of haloacetaldehydes with cAMP. Each haloacetaldehyde was reacted at its own pH optimum as described in the text. ○, Bromoacetaldehyde at 100°C; ●, bromoacetaldehyde at 80°C; □, iodoacetaldehyde at 80°C; ■, chloroacetaldehyde at 100°C.

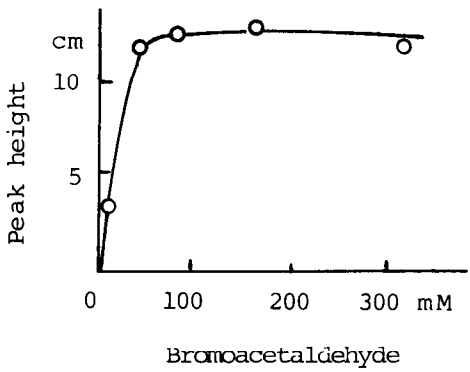

Fig. 5. Reactions of various concentrations of bromoacetaldehyde with cAMP at pH 5.

A dose-dependent reaction with bromoacetaldehyde showed a plateau at 90 m*M* at pH 5 as shown in Fig. 5, or at 170 m*M* at pH 5. ATP and ADP must be reacted at pH 7, since the pyrophosphate linkage was hydrolysed to some extent at pH 5. Two standard reaction conditions were made as described under Quantitative reaction. The reaction products were stable for as long as fifteen days when stored at 4°C (data not shown).

Working curves of the adenine nucleotides were linear for injected amounts between 1 and 160 pmol (data not shown).

The established method was applied to the measurement of cAMP in human urine after clean-up. In the chromatograms, deoxy cAMP (as internal standard) and cAMP appeared and were followed by low peaks of unknown compounds as shown in Fig. 6. To separate deoxy cAMP from cAMP completely, the concentration of sodium chloride in the eluent had to be decreased to 0.3 *M*. The values obtained with HPLC were compared with those obtained by RIA. The good correlation is shown in Fig. 7.

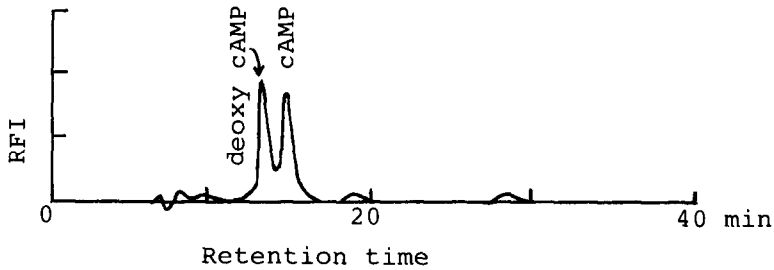

Fig. 6. Chromatogram of human urine. The amount is in the range as shown in Fig. 7.

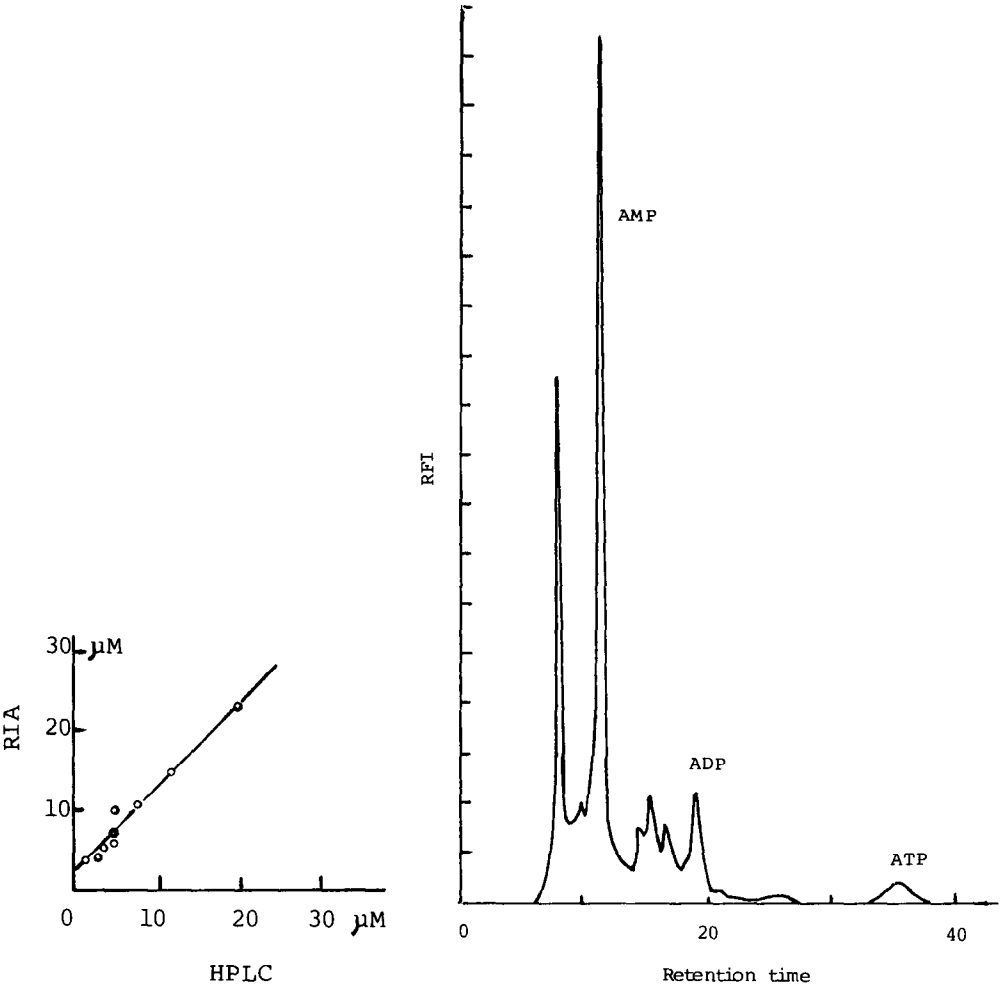

Fig. 7. Correlation between concentrations of cAMP in urine determined by HPLC and RIA. The concentration of cAMP was measured by RIA [1]. $Y = 1.07X + 2.25$ ($r = 0.98$).

Fig. 8. Chromatogram of rat brain. The amounts are described in the text.

The adenine nucleotides in rat brain were determined as shown in Fig. 8. The amounts of AMP, ADP and ATP were 68, 115 and 419 nmol/g wet weight, respectively. The recovery of the adenine nucleotides, each added with 0.4 *M* perchloric acid, was 100% from brain or blood.

ADP and ATP in plasma are shown in Fig. 9. Their concentrations were 13 and 28 μM, respectively. Their concentration in serum was very low, the concentration of ATP being 2—3 μM.

To obtain a higher sensitivity, the fluorescence spectrophotometer shown in Fig. 1 was constructed. It was more efficient to make the light of 253.7 nm from the low-pressure mercury lamp monochromatic with the concave grating than with the regular interference filter. The concave grating of F 2.5 used was the brightest in this kind. The lamp and flow-through cell could be used with other fluorescence systems. The sensitivity was extremely high, as shown in Fig. 10. Femtomole amounts of adenine nucleotides were quantitatively separated by micro-HPLC with the spectrophotometer.

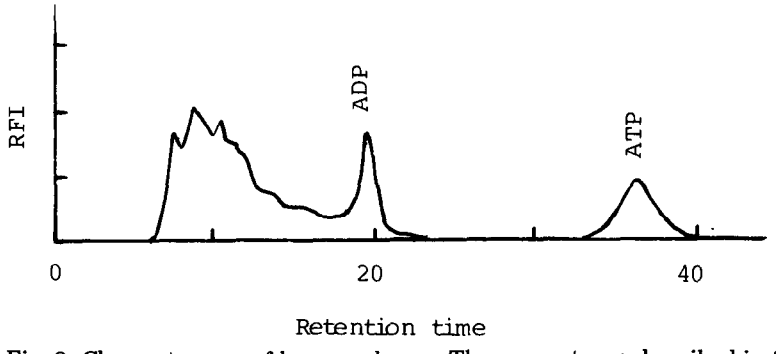

Fig. 9. Chromatogram of human plasma. The amounts are described in the text.

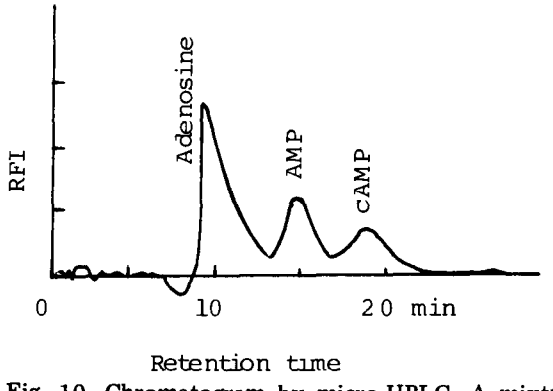

Fig. 10. Chromatogram by micro-HPLC. A mixture of the compounds (20 fmol of each was injected.

DISCUSSION

It was established that the adenine compounds could be determined simultaneously by HPLC with the new fluorescence reagent. The reaction of bromoacetaldehyde with adenine compounds was completed in a short time, and was reproducible because of the high yield and stability of the fluorescence products. The reaction of bromoacetaldehyde with nucleic acid bases was as selective as with chloroacetaldehyde except the reaction with guanine base, which will be described elsewhere. Taking the high resolution of HPLC into consideration, the method was made selective for the adenine compounds. It

was relevant for reliable determination to see the peaks of ADP and ATP in biological samples as shown in Figs. 8 and 9.

These compounds have been separated by inconvenient gradient elution HPLC assays [3–6, 14] which are not selective and sensitive. They have also been determined by an enzymatic method with luciferase [15], which is troubled with a high background. The present method is sensitive enough to measure cAMP in urine. The values obtained were compatible with those obtained by RIA as shown in Fig. 7, but the present method does not involve the care required in handling radioisotopes.

Further, the method is widely applicable to the measurement of adenine compounds in various samples. We have already described substantial findings such as adenosine in synaptosomes from guinea pig cerebral cortex [16] and adenine nucleotide release with catecholamines from adrenal chromaffin cells [17].

The applicability of the method to other adenine compounds can be widened by changing the separation system. Micro-HPLC as shown in Fig. 10 is also promising to cope with minute volumes of important biological samples.

Thus, the method described is useful for the exploration of adenine compounds in basic research and clinical fields.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research No. 487145 from the Ministry of Education, Science and Culture, and a grant from the Iatrochemical Foundation, for which the authors' thanks are due. We are deeply grateful to Mr. K. Suga of Daiichi Pure Chemicals Co. for measuring cAMP by RIA. We appreciate that Hitachi Ltd., Tokyo, supplied us with the Hitachi gel No. 3012-N under investigation.

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