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Note

Determination of sodium N^6,2′-*O*-dibutyryladenosine cyclic 3′,5′-(hydrogen phosphate) and its hydrolysis products by high-performance liquid chromatography

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DBcAMP*, a biologically active nucleotide (Fig. 1), hydrolyzes readily in aqueous solution into cAMP via 2′-O-MBcAMP and/or N^6-MBcAMP. The kinetics of the hydrolysis reaction has not been examined in detail. Only a column chromatographic method has been presented for the determination of DBcAMP and its hydrolysis products[1]; however, the method is tedious and time-consuming for use in routine work. In order to clarify the kinetics of hydrolysis of DBcAMP a more efficient and reliable method was required.

This paper presents a simple and precise method for the simultaneous determination of DBcAMP, 2′-O-MBcAMP, N^6-MBcAMP and cAMP by high-performance liquid chromatography (HPLC).

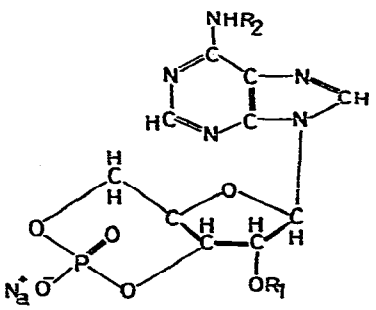

Fig. 1. Structures of DBcAMP and its hydrolysis products.
DBcAMP: $R_1 = R_2 = COC_3H_7$
2′-O-MBcAMP: $R_1 = COC_3H_7$, $R_2 = H$
N^6-MBcAMP: $R_1 = H$, $R_2 = COC_3H_7$
cAMP: $R_1 = R_2 = H$

* Abbreviations: DBcAMP = sodium N^6,2′-O-dibutyryladenosine cyclic 3′,5′-(hydrogen phosphate); 2′-O-MBcAMP = sodium 2′-O-monobutyryladenosine cyclic 3′,5′-(hydrogen phosphate); N^6-MBcAMP = sodium N^6-monobutyryladenosine cyclic 3′,5′-(hydrogen phosphate); cAMP = sodium adenosine cyclic 3′,5′-(hydrogen phosphate).

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EXPERIMENTAL

Materials and reagents

DBcAMP, 2′-O-MBcAMP, N^6-MBcAMP and cAMP were synthesized in our Research Institute. Acetonitrile, specially prepared for HPLC (Nakarai Chemicals, Kyoto, Japan) and acetic acid of analytical reagent grade were used. Water was distilled and all other chemicals were of reagent grade. A column (150 × 6 mm I.D.) packed with Develosil ODS (particle size 5 μm; Nomura Kagaku, Aichi, Japan) was used. It was prepared by a slurry method with slurry solvent B (Machery, Nagel & Co., Düren, G.F.R.). Acetonitrile–1 % acetic acid (30:70) was used as mobile phase.

Apparatus

An Hitachi 638 liquid chromatograph equipped with a variable-wavelength UV-detector UVILOG-5II (Oyobunko, Tokyo, Japan) and Rheodyne 7122 sample injector with 20-μl sampling loop (Rheodyne, CA, U.S.A.) was used. The UV detector was set at 273 nm and 0.08 a.u.f.s.

Ananalytical procedure

Samples were dissolved in the mobile phase to give solutions in the range of about 2–40 μg ml^{-1} for DBcAMP, 2′-O-MBcAMP, N^6-MBcAMP and cAMP. The sample solutions were analyzed by HPLC at a mobile phase flow-rate of 1.0 ml min^{-1}. The concentrations of each compound in the samples were determined from calibration curves prepared by using the peak height method.

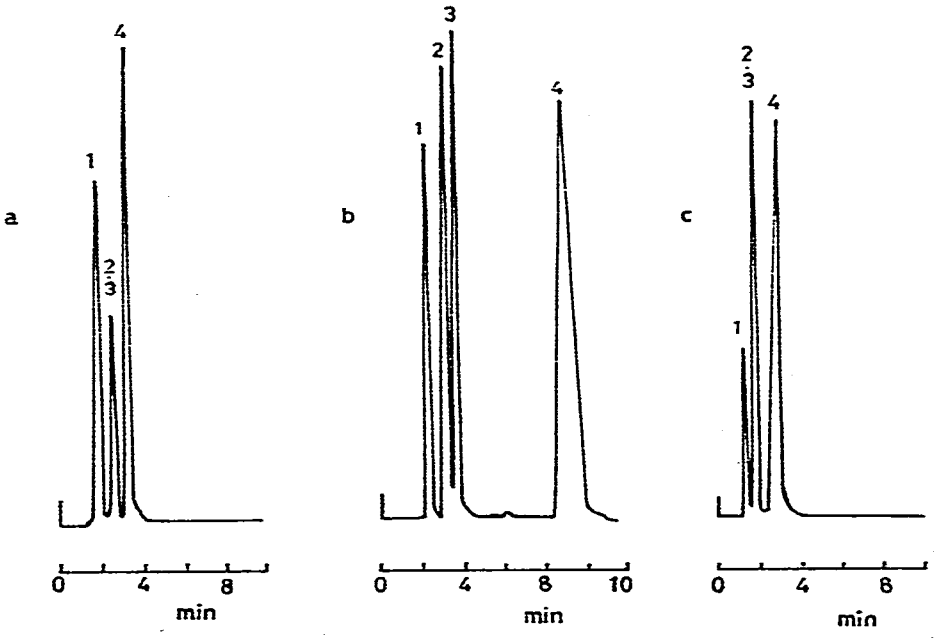

Fig. 2. Separation of DBcAMP and its degradation products using the stationary phases Nucleosil 5 C_{18} (a), Develosil 5 ODS (b) and Cosmosil 5 C_{18} (c). Peaks: 1 = cAMP; 2 = 2′-O-MBcAMP; 3 = N^6-MBcAMP; 4 = DBcAMP.

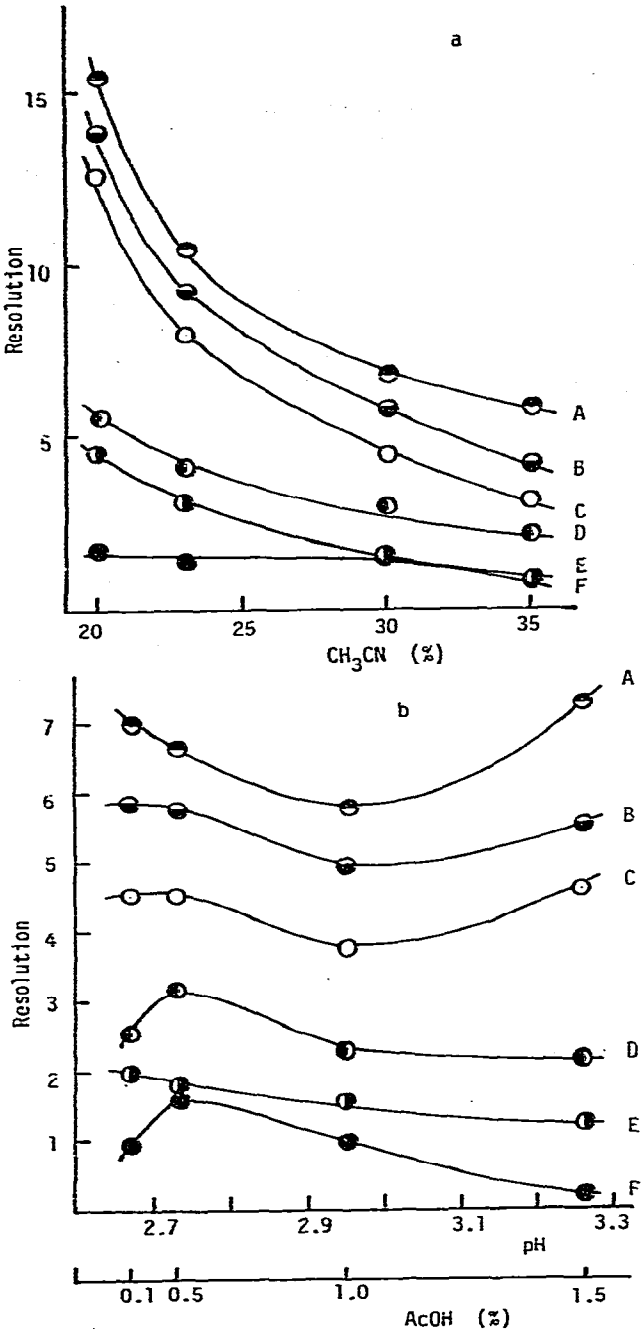

Fig. 3. Influence of acetonitrile and acetic acid (AcOH) concentrations in the mobile phase on mutual resolution between peaks 1 (cAMP), 2(2′-O-MBcAMP), 3(N^6-MBcAMP) and 4(DBcAMP). (a) Acetonitrile concentration in 1 % acetic acid; (b) acetic acid concentration in 30 % acetonitrile A, peaks 1 and 4; B, peaks 2 and 3; C, peaks 3 and 4; D, peaks 1 and 3; E, peaks 1 and 2; F, peaks 2 and 3. Column: Develosil 5 ODS (150 × 6.0 mm I.D.).

RESULTS AND DISCUSSION

Choice of stationary phase and column size

Preliminary attempts to separate DBcAMP and its hydrolysis products were made with three kinds of reversed-phase materials, Develosil ODS, Nucleosil C_{18} (particle size 5 μm, Macherey, Nagel & Co.) and Cosmosil C_{18} (particle size, 5 μm Nakarai Chemicals), in a column of 150 × 4.6 mm I.D. The numbers of theoretical plates, N, of these columns, which were calculated by the usual way[2] from the elution pattern of anthracene using methanol–water (80:20), were similar (N = 6500 for Develosil ODS, N = 6800 for Nucleosil C18, N = 6300 for Cosmosil C18), whereas significant differences were found for separations between DBcAMP and its hydrolysis products as shown in Fig. 2. The most favourable separation was observed on the reversed-phase of Develosil ODS, however it was considered to be not sufficient for the simultaneous determination of DBcAMP and its hydrolysis products. When using a Develosil column of 150 × 6 mm I.D., a more efficient separation was achieved.

Influence of mobile phase

In order to obtain the optimum separation on the Develosoil ODS column (150 × 6 mm I.D.), the influence of the mobile phase was examined with the solvent system of acetonitrile–water–acetic acid. Fig. 3 shows the influence of the concentrations of acetonitrile (a) and acetic acid (b) in on the resolution (R_s) between any two compounds. Each R_s value was calculated in the usual way[3].

In general, efficient and selective chromatographic analyses have been run at separation 6σ corresponding to R_s 1.5. As seen in Fig. 3, a decrease in acetonitrile

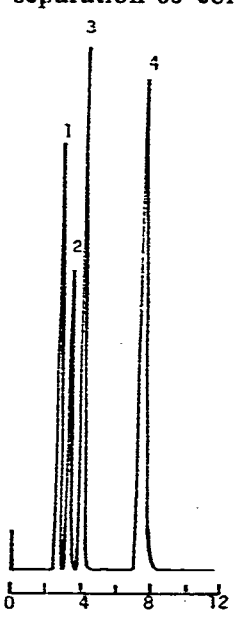

Fig. 4. Typical separation of DBcAMP and its hydrolysis products. Stationary phase: Develosil 5 ODS, 150 × 6.0 mm I.D. Mobile phase: acetonitrile–1 % acetic acid (30:70). Peaks as in Fig. 2.

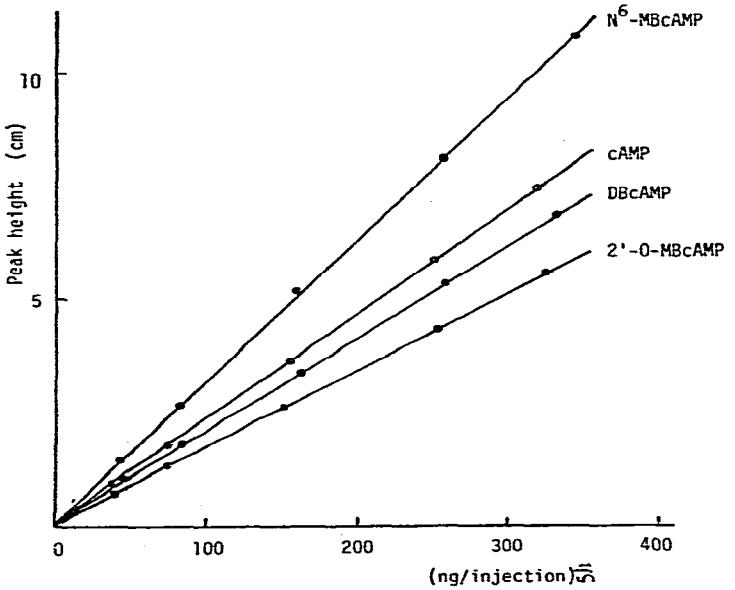

Fig. 5. The calibration curves of DBcAMP and its hydrolysis products. Each peak height is the mean from two determinations.

concentration results in greater R_s for any two nucleotides, however this required a longer analysis time. The optimum concentration of acetonitrile was considered to be 30% from the viewpoint of sufficient separation and length of the analysis time. On the other hand, the influence of acetic acid on R_s is more complicated. A mobile phase of acetonitrile–1% acetic acid (30:70) gave the maximum R_s (1.7) between N^6-MBcAMP and 2′-O-MBcAMP, and this was used for subsequent experiments.

Monitoring wavelength

The UV absorption spectra of the nucleotides in the mobile phase exhibited λ_{max} at 273 nm for DBcAMP and N^6-MBcAMP, and at 257 nm for 2′-O-MBcAMP and cAMP, so the detection was carried out by monitoring the absorbance at 273 nm.

Calibration curves and precision

Fig. 4 shows a typical elution pattern of DBcAMP and its hydrolysis products, and Fig. 5 shows the corresponding calibration curves. The calibration curves were linear. The reproducibility among runs was about 2% or below of the coefficient of variation. The detection limit of each compound was about 1 ng per injection.

The method presented was used successfully for a kinetic study of the hydrolysis of DBcAMP. The results will be presented in a later paper.

REFERENCES

1 F. A. Neelon, B. M. Birch and H. E. Lebovitz, *Anal. Biochem.*, 56 (1973) 74.
2 F. Bauman, *Basic Liquid Chromatography*, Varian Aerograph, Walnut Creek, CA, 1971.
3 J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley, New York, 1971.