Journal of Chromatography, 196 (1980) 323–330
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,870

Note

Control of 8-bromo-AMP and 8-(6-aminohexyl)amino-AMP synthesis by high-performance liquid chromatography

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(Received April 4th, 1980)

8-Bromo-AMP and 8-(6-aminohexyl)amino-AMP are intermediates in the synthesis of agarose-immobilized adenosine nucleotides suitable for affinity chromatography[1–3]. The time course of the reaction is generally followed by ultraviolet or nuclear magnetic resonance spectroscopy, which do not allow the quantitative determination of the yield of the reaction. The reaction products are usually checked by paper electrophoresis or paper chromatography.

High-performance liquid chromatography (HPLC) is now a method of choice for the determination of nucleotides[4,5]. Isocratic separation of simple nucleotide mixtures has been reported on ion-exchange[6,7] and reversed-phase[8,9] columns. However, the resolution of more complex mixtures requires the use of gradient elution[10–13].

We report here the rapid separation of AMP, 8-bromo-AMP and 8-(6-aminohexyl)amino-AMP by HPLC on ion-exchange and reversed-phase columns. This technique allows the monitoring of the reaction time course during the synthesis of the AMP derivatives and the precise and rapid determination of the purity of the reaction products.

MATERIALS AND METHODS

Chemicals

AMP and 8-(6-aminohexyl)amino-AMP were purchased from Sigma (St. Louis, MO, U.S.A.). 8-Bromo-AMP was synthesized according to Lee *et al.*[1]. Tetrabutylammonium perchlorate was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). All other chemicals were analytical-reagent grade materials from Merck (Darmstadt, G.F.R.).

The nucleotide standards (80–100 μM) were prepared in distilled water and stored at −20°C.

Apparatus

A Chromatem 38 liquid chromatograph (Touzart & Matignon, Paris, France) was used. It was fitted with a double-beam UV detector operating at 254 nm and equipped with an 8-μl cell. A loop injector was used for the injection of 10-μl samples.

0021-9673/80/0000–0000/$02.25

Columns

A Partisil 10-SAX ion-exchange column from Whatman (Clifton, NJ, U.S.A.) was employed. Before use, the column (20 cm × 4.6 mm I.D.) was equilibrated with the buffer during 60 min.

For reversed-phase liquid chromatography two columns were used. For classical reversed-phase chromatography a Nucleosil C_{18} column (15 cm × 4.6 mm I.D.) from Macherey, Nagel & Co. (Düren, G.F.R.), particle size 5 μm, was used, and for ion-pair reversed-phase chromatography a Zorbax ODS column (28 cm × × 4.6 mm I.D.) from DuPont (Wilmington, DE, U.S.A.), particle size 5 μm, was used. These columns were washed for 15 min with the buffer before use. The columns were packed by a slurry packing technique[14].

Preparation of buffers

Buffer A. This buffer was used for the ion-exchange column and consisted of 12 m*M* Na_2HPO_4 adjusted to the desired pH with 6 m*M* citric acid.

Buffer B. This buffer was used for reversed-phase chromatography on Nucleosil C_{18} and consisted of 0.1 *M* sodium phosphate buffer (pH 6.5) containing 10% (v/v) of methanol.

Buffer C. This buffer was used for ion-pair reversed-phase chromatography on Zorbax ODS and consisted of a 2:1 mixture of 30 m*M* KH_2PO_4 in water and 9 m*M* tetrabutylammonium perchlorate in methanol[15]. The pH of the mixture was adjusted with potassium hydroxide or phosphoric acid.

The buffers were filtered on 0.45-μm Sartorius filters and degassed by sonication before use.

Quantitation of peaks

A calibration run was performed with known amounts of each compound and the peaks were quantitated using the peak height.

Synthesis of 8-bromo-AMP

8-Bromo-AMP was synthesized according to Lee *et al.*[1] AMP (20 mg) was dissolved in 1 ml of 1 *M* acetate buffer (pH 3.9), 10 μl of liquid bromine were added and the solution was stirred vigorously at room temperature in the dark. At different time intervals, 1-μl aliquots were taken, diluted with 400 μl of buffer A and injected directly on to the ion-exchange column.

Synthesis of 8-(6-aminohexyl)amino-AMP

8-(6-Aminohexyl)amino-AMP was synthesized according to Lee *et al.*[1]. 8-Bromo-AMP (50 mg) was dissolved in 500 μl of water and mixed with 500 mg of hexamethylenediamine dissolved in 500 μl of water. The solution was adjusted to pH 8.9 with hydrochloric acid and incubated at 99°C. At different time intervals, 1-μl aliquots were taken, diluted with 500 μl of buffer A and injected directly on to the ion-exchange column.

RESULTS AND DISCUSSION

Reversed-phase chromatography

The separation of AMP and 8-bromo-AMP by classical reversed-phase chro-

matography on the Nucleosil C_{18} column is shown in Fig. 1. 8-(6-Aminohexyl) amino-AMP did not elute from the column in a reasonable time. Increasing the concentration of methanol resulted in a poor separation of AMP and 8-bromo-AMP.

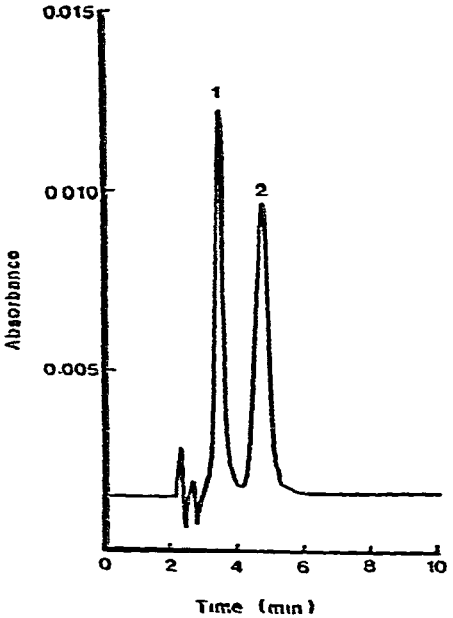


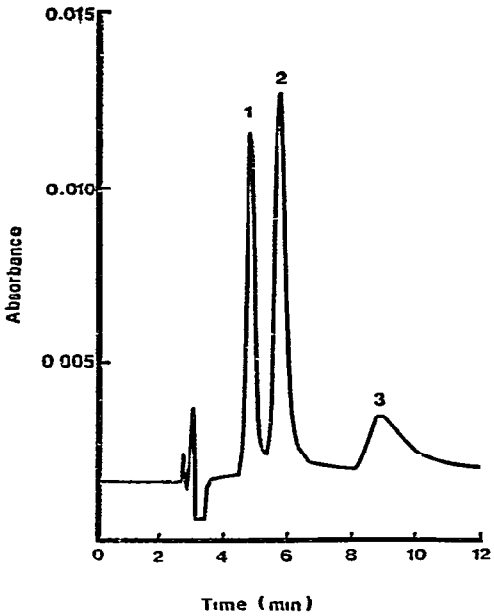
Fig. 1. Separation of AMP (1) and 8-bromo-AMP (2) by reversed-phase chromatography. Column, 15 cm × 4.6 mm I.D., Nucleosil C_{18}, 5 μm; eluent, 0.1 *M* NaH_2PO_4 buffer (pH 6.5)–10% methanol; flow-rate, 1 ml/min; pressure, 1900 p.s.i.; temperature, ambient; sample volume, 10 μl; detection, 254 nm.

Fig. 2 shows the separation of the three compounds by ion-pair reversed-phase chromatography on the Zorbax ODS column. A good separation was achieved. However, spreading of the 8-(6-aminohexyl)amino-AMP peak was observed. Fig. 3 shows the variation of the retention times with the pH of the eluent. Whereas the retention times of AMP and 8-bromo-AMP were hardly affected by changes in pH, the retention time of 8-(6-aminohexyl)amino-AMP decreased with pH. This effect is probably related to the ionization of the ω-NH_2 group of the aminohexyl moiety. Hence a good separation can be achieved as a consequence of the introduction of a positive charge which is not neutralized by the ion-pairing agent and which compensates the hydrophobicity of the alkyl chain.

Ion-exchange chromatography

As shown in Fig. 4, a good separation of the three compounds was achieved at pH 7.0. The order of elution was the same as in reversed-phase chromatography.

The alkyl chains forming the quaternary ammonium, which is the functional group of Partisil 10-SAX, could induce reversed-phase behaviour under some conditions as shown in Fig. 5. Methanol lowers the retention time of 8-(6-aminohexyl)-



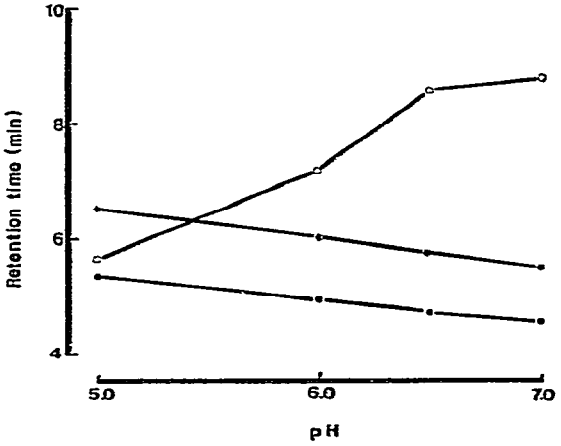


Fig. 2. Separation of AMP (1), 8-bromo-AMP (2) and 8-(6-aminohexyl)amino-AMP (3) by ion-pair reversed-phase chromatography. Column, 28 cm × 4.6 mm I.D., Zorbax ODS, 5 μm; eluent, 2:1 mixture of 30 m*M* KH_2PO_4 in water and 9 m*M* tetrabutylammonium perchlorate in methanol (pH 7.0); flow-rate, 0.7 ml/min; pressure, 1500 p.s.i.; temperature, ambient; sample volume, 10 μl; detection, 254 nm.

Fig. 3. Retention times of AMP (●), 8-bromo-AMP (✱) and 8-(6-aminohexyl)amino-AMP (○) as a function of pH. Operating conditions as in Fig. 2.

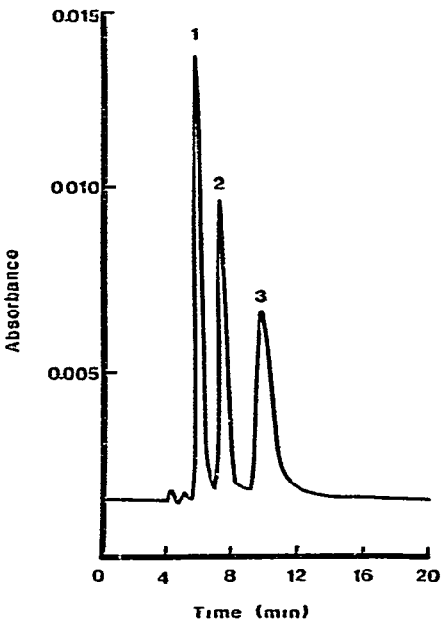


Fig. 4. Separation of AMP (1), 8-bromo-AMP (2) and 8-(6-aminohexyl)amino-AMP (3) by ion-exchange chromatography. Column, 20 cm × 4.6 mm I.D., Partisil 10-SAX, 10 μm; eluent, 12 m*M* Na_2HPO_4–6 m*M* citric acid (pH 7.0); flow-rate, 0.73 ml/min; pressure, 290 p.s.i.; temperature, ambient; sample volume, 10 μl; detection, 254 nm.

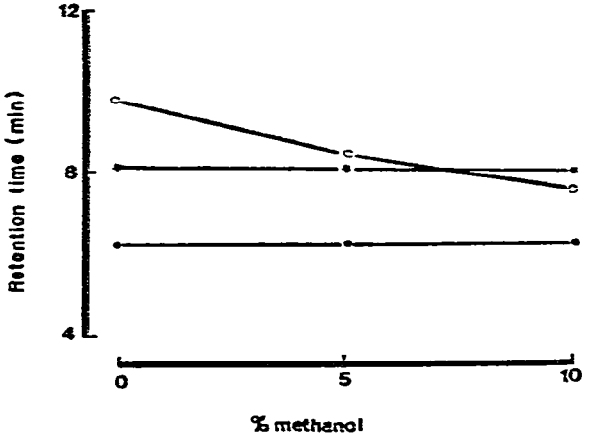


Fig. 5. Retention times of AMP (●), 8-bromo-AMP (★) and 8-(6-aminohexyl)amino-AMP (○) as a function of methanol concentration. Operating conditions as in Fig. 4.

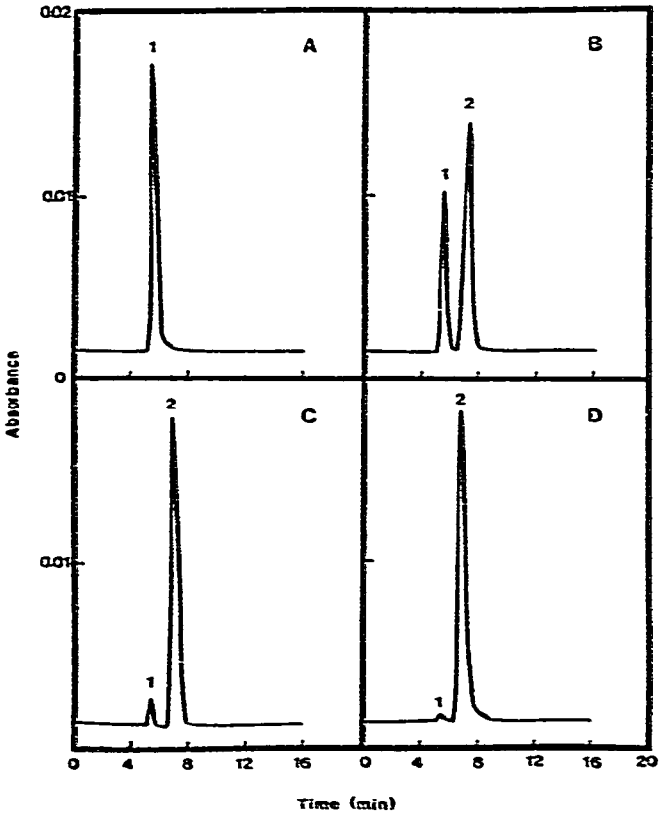


Fig. 6. Progressive formation of 8-bromo-AMP from AMP displayed by ion-exchange chromatography. Aliquots of 1 μl of the reaction mixture (see Materials and methods) were diluted with 800 μl of buffer A at time 0 (A), or with 400 μl of buffer A at times 5 min (B), 30 min (C) and 120 min (D), and injected on to the column. Chromatographic conditions as in Fig. 4.

amino-AMP but not those of AMP and 8-bromo-AMP. Hence for the first compound Partisil 10-SAX behaves like a mixed system, showing both ion-exchange and reversed-phase properties.

Control of 8-bromo-AMP synthesis

Fig. 6 shows the appearance of 8-bromo-AMP with time. The overall yield of the reaction was high, reaching 96% after 60 min (Fig. 7). The reaction occurred until exhaustion of the AMP and a 100% yield was obtained after 2 h.

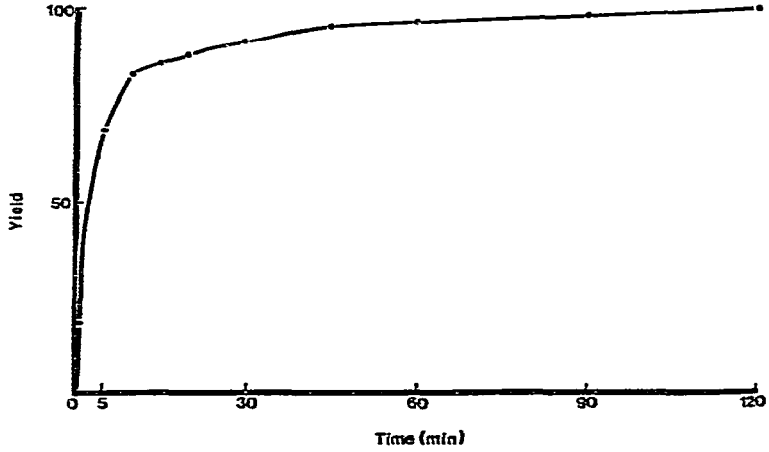


Fig. 7. Yield of 8-bromo-AMP synthesis as a function of reaction time. Conditions as in Fig. 6.

The repeated injection of dilute bromine did not alter the properties of the column. Hence in this instance HPLC is an easy and rapid method for the monitoring of the reaction time course.

Control of 8-(6-aminohexyl)amino-AMP synthesis

Fig. 8 shows the appearance of 8-(6-aminohexyl)amino-AMP with time. The 8-bromo-AMP used was contaminated with about 1.5% of AMP. This AMP concentration increased to about 2.8% of the total amount of nucleotides after 2–3 h, indicating slight hydrolysis of 8-bromo-AMP, and then decreased to about 2% after 10 h.

The sum of the three nucleotides, after 10 h, represents 75% of the initial concentration, probably indicating a degradation to nucleosides. The maximal reaction yield (70%) was obtained after 6 h (Fig. 9).

In conclusion, the speed of analysis by HPLC allows the control of the time course and yield of the synthesis of 8-bromo-AMP and 8-(6-aminohexyl)amino-AMP. The method also allows the rapid determination of the purity of the reaction products. It is likely that these techniques can be used, with minor modifications, for the separation of the corresponding diphosphate and triphosphate nucleotide analogues commonly prepared for affinity chromatography.

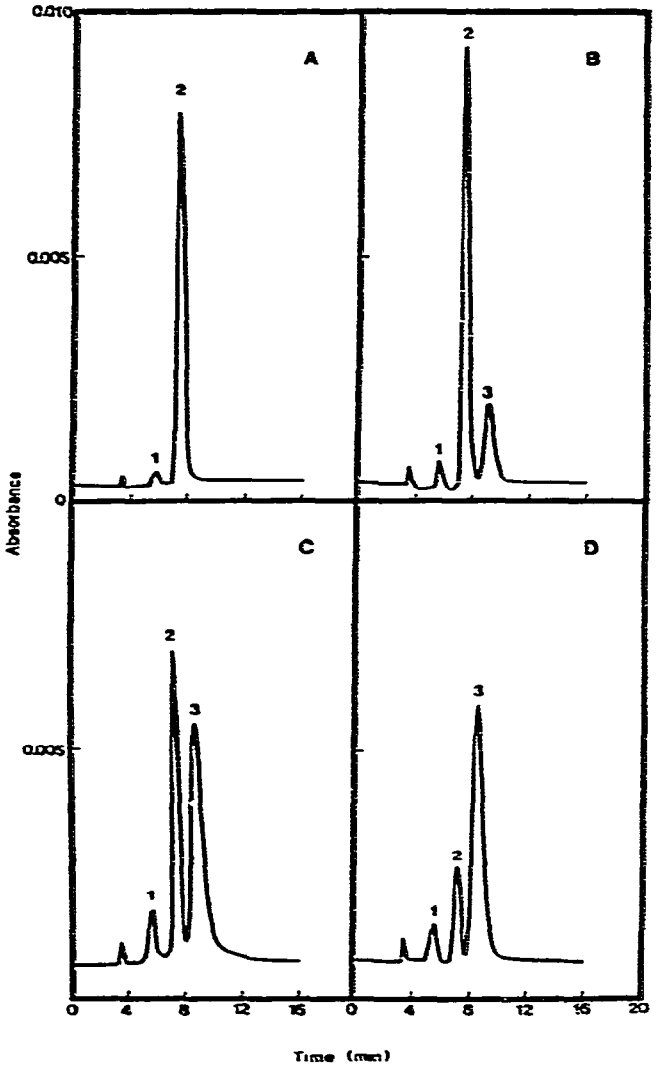


Fig. 8. Progressive formation of 8-(6-aminohexyl)amino-AMP from 8-bromo-AMP displayed by ion-exchange chromatography. Aliquots of 1 μl of the reaction mixture (see Materials and methods) were diluted with 1 ml of buffer A at time 0 (A) or with 500 μl of buffer A at times 1 h (B), 3 h (C) and 6 h (D), and injected on to the column. Chromatographic conditions as in Fig. 4.

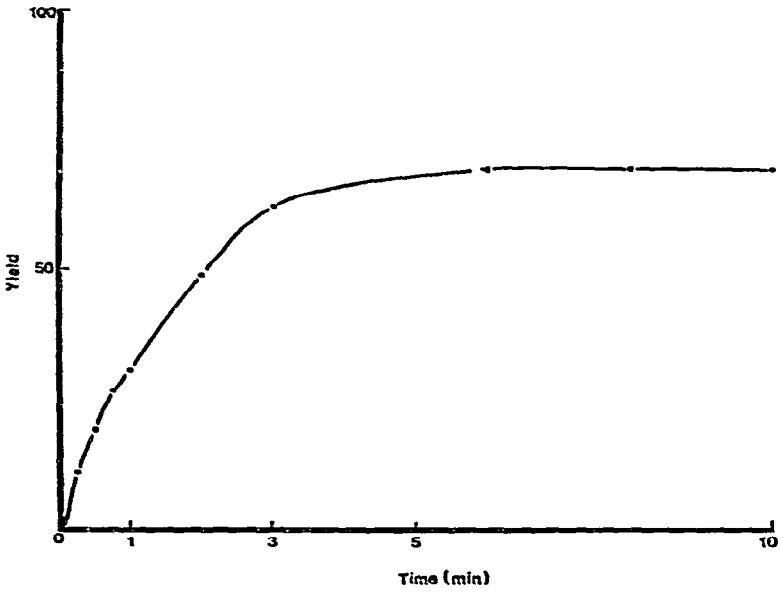


Fig. 9. Yield of 8-(6-aminohexyl)amino-AMP synthesis as a function of reaction time. Conditions as in Fig. 8.

ACKNOWLEDGEMENT

The authors are grateful to Mrs. G. Gutbier for technical assistance.

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