Journal of Chromatography, 258 (1983) 199–205
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,513

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SOME AMP DERIVATIVES USED AS AFFINITY LIGANDS

J. KOVÁŘ*
Department of Biochemistry, Purkynje University, Kotlářská 2, 611 37 Brno (Czechoslovakia)
V. KAHLE
Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, Leninova 82, 611 42 Brno (Czechoslovakia)
and
J. TURÁNEK
Department of Biochemistry, Purkynje University, Kotlářská 2, 611 37 Brno (Czechoslovakia)
(Received November 9th, 1982)

SUMMARY

Separation of AMP, N^1-carboxymethyl-AMP, N^6-carboxymethyl-AMP and N^6-[N-(6-aminohexyl)carbamoylmethyl]-AMP by reversed-phase liquid chromatography is described. A 25 m*M* sodium phosphate buffer (pH 2.5–5.0) proved to be the most suitable mobile phase for the separation of AMP and the intermediates, while for the elution of the final product 10% methanol in the buffer (pH 2.5) gave satisfactory results. Identification of the peaks was performed by means of ultraviolet and infrared spectroscopic measurements, by cyclic voltammetry and by reaction with ninhydrin. Analysis of the reaction mixtures at successive stages of the synthesis of N^6-[N-(6-aminohexyl)carbamoylmethyl]-AMP by high-performance liquid chromatography enabled us to propose two modifications of the separation procedure of intermediates and final product.

INTRODUCTION

Several derivatives of 5′-adenosine monophosphate (5′-AMP) are frequently used as general ligands in the affinity chromatography of dehydrogenases and other enzymes[1]. N^6-substituted AMP analogues, *e.g.* N^6-carboxymethyl-AMP (compound II) and N^6-[N-(6-aminohexyl)carbamoylmethyl]-AMP (compound III), belong to the most important affinants which are often immobilized to Sepharose, cellulose and other supports. Compound III bound to Sepharose 4B is commercially available (*e.g.* from Pharmacia, Uppsala, Sweden). The course of the synthesis of III (starting from AMP) is usually followed by thin-layer chromatography[2]. However, recent papers concerning the analysis of AMP and similar compounds suggest that high-performance liquid chromatography (HPLC) on weak anion exchangers or on a reversed phase is the most efficient method for the separation of these compounds[3].

0021-9673/83/0000–0000/$03.00

The aim of this paper is to show the possibility of monitoring the consecutive steps of the synthesis of II and III by HPLC on a C_{18} reversed-phase and to demonstrate the contribution of HPLC analysis to improving the preparative separation of these derivatives. An analogous problem, *i.e.* the monitoring of the synthesis of similar NAD derivatives from NAD, has already been dealt with[4].

EXPERIMENTAL

Materials

AMP was obtained from Reanal (Budapest, Hungary), iodoacetic acid (Lachema, Brno, Czechoslovakia) was purified by crystallization from light petroleum (b.p. 60–80°C), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (referred to as carbodiimide) was the product of Sigma (St. Louis, MO, U.S.A.), Dowex 1-X2 was purchased from Dow Chemical (Midland, MI, U.S.A.). Other chemicals were of analytical-reagent grade.

Synthesis of N^6-[N-(6-aminohexyl)carbamoylmethyl]-AMP (III)

This compound was prepared according to ref. 5; the principle of the synthesis is shown in Fig. 1. First, AMP was alkylated by an excess of iodoacetic acid yielding N^1-carboxymethyl-AMP (I). This compound was rearranged in alkaline medium to N^6-carboxymethyl-AMP (II). Thereafter, condensation of II with 1,6-diaminohexane in the presence of carbodiimide was performed. The intermediates and the final poduct III were purified as described in ref. 5. For the analysis of the course of these three reactions we used AMP and the compounds I and II purified by an additional precipitation with cold ethanol.

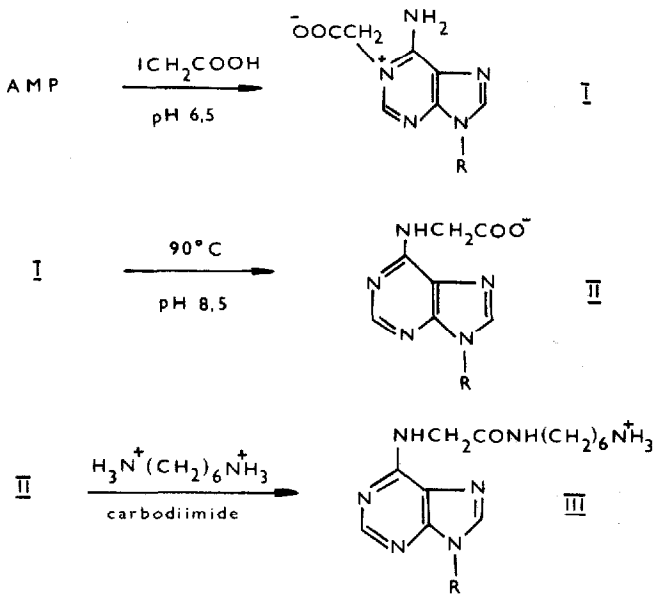

Fig. 1. Synthesis of N^6-[N-(6-aminohexyl)carbamoylmethyl]-AMP (III). Reaction conditions were identical with those described in ref. 5. R denotes phosphoribosyl (the unchanged moiety of AMP).

HPLC separation

The liquid chromatograph consisted of a MC 100 pump (Mikrotechna, Czechoslovakia) home-made stainless steel columns and UV 254 detector (GSAV workshops, Czechoslovakia). Columns of dimensions 150 × 4 mm (for analytical) and 250 × 6.5 mm (for semi-preparative separations) were packed with Separon Si C_{18} (5 μm) (Laboratory Instruments, Czechoslovakia) by the viscosity method[6]. The mobile phase used was 25 m*M* sodium phosphate buffer (pH 2.5–8.0) or the same buffer containing 4–50% methanol. Samples were injected with an HP-305 Hamilton syringe through a silicon septum. A pump was used for the loading of the sample on the semi-preparative column.

Identification of the compounds

Fractions eluted from the HPLC column were brought to pH 7 by addition of 0.5 *M* sodium phosphate buffer (pH 7) and characterized by their UV and IR spectra, by cyclic voltammograms and by reaction with ninhydrin. UV–visible spectroscopic measurements were performed in a Cary 118 spectrophotometer (Varian). IR spectra were measured with solid compounds obtained by precipitation with ethanol in KBr pellets using a UR-20 IR spectrometer (Zeiss). A PRG-4 polarograph (Tacussel) was used for voltammetric measurements.

RESULTS AND DISCUSSION

HPLC separation of intermediates and products

A typical chromatogram obtained for a reaction mixture after carboxymethylation of AMP is shown in Fig. 2A. The lower capacity factor of I, compared with that of AMP, can be caused by the presence of a charged nitrogen in I (*cf.* Fig. 1). A similar effect was observed for N^1-carboxymethyl-NAD[4]. During this reaction step a small amount of the rearranged product II is formed as well (see below and *cf.* Fig. 2A). The reaction course of the carboxymethylation monitored by HPLC is dem-

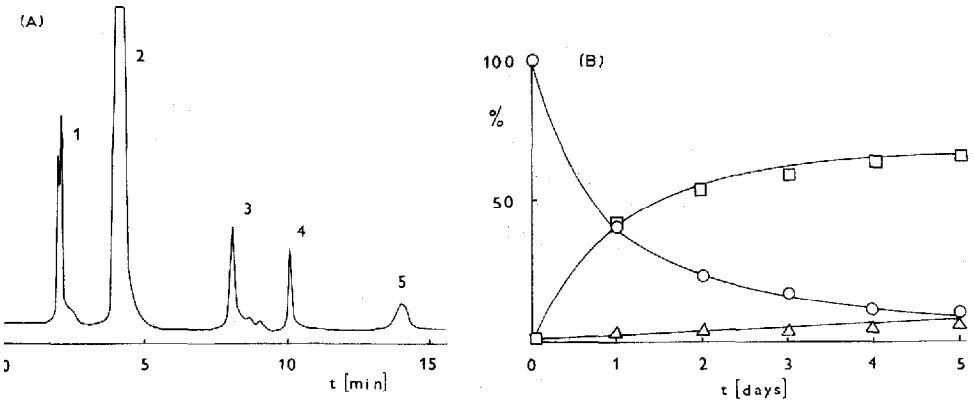

Fig. 2. Carboxymethylation of AMP. (A) Separation of the reaction mixture. Column, Separon C_{18}; mobile phase, 25 m*M* phosphate buffer, pH 2.5; flow-rate, 0.75 ml/min; pressure, 4.5 mPa. Peaks: 1 = iodide and unknown (λ_{max} = 272 nm); 2 = N^1-carboxymethyl-AMP (I); 3 = AMP; 4 = iodoacetic acid; 5 = N^6-carboxymethyl-AMP (II). (B) Reaction course monitored by HPLC. ○ = AMP; □ = I; △ = II. Results are expressed as percentages of the initial AMP concentration.

onstrated in Fig. 2B. The yields of I and II are approximately 65 and 5–10%, respectively; *ca.* 20% of undesirable by-products are formed and nearly 10% of unmodified AMP remains in the reaction mixture under the conditions described[5]. Nearly one half of the by-products are polar compounds of very low retention (Fig. 2A) with an absorption maximum at 272 nm, the greater part of the by-products being red compounds (λ_{max} = 510 nm) which are bound very tightly to the reversed phase and cannot be eluted from the column even by methanol or ethanol.

Fig. 3A shows a chromatogram of the reaction mixture after the rearrangement. Like N^6-carboxymethyl-NAD[4], compound II has a greater retention than AMP and I. The rearrangement of I into II is nearly complete (>90%) and the small amount of AMP contained in the reaction mixture remains unchanged during this reaction (*cf.* Fig. 3B). The conditions used for this reaction also lead to the formation of red by-products with high retention (λ_{max} = 510 nm) and of other minor by-products.

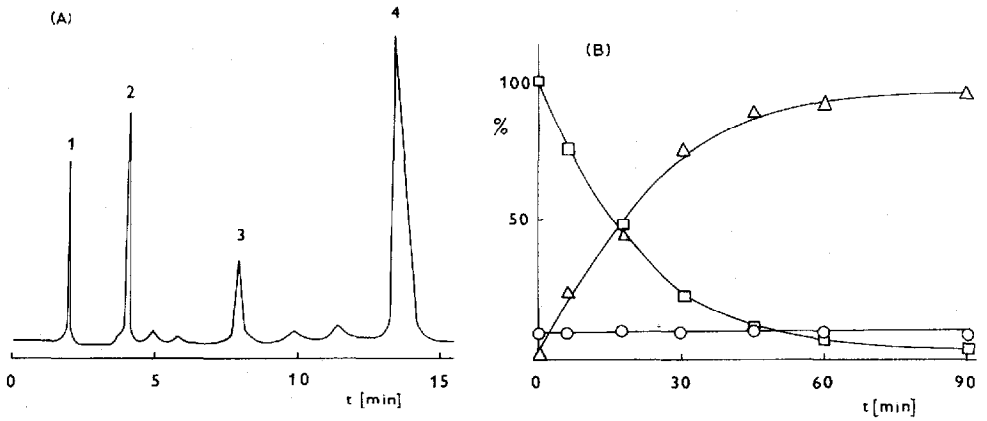

Fig. 3. Rearrangement of N^1-carboxymethyl-AMP. (A) Separation of the reaction mixture. Operating conditions the same as in Fig. 2A. Peaks: 1 = unknown (λ_{max} = 272 nm); 2 = N^1-carboxymethyl-AMP (I); 3 = AMP; 4 = N^6-carboxymethyl-AMP (II). (B) Reaction course monitored by HPLC; symbols as in Fig. 2B. Results are expressed as percentages of the initial concentration of I.

The incorporation of the aminohexyl group into the molecule of II brings about a substantial increase in the retention of the resulting compound III. Phosphate buffer (pH 2.5) containing 10% methanol was found to be suitable for the elution of III from the reversed phase (Fig. 4A). The yield of III is *ca.* 75% (*cf.* Fig. 4B); *ca.* 10% of II remains unchanged under the conditions applied[5]. The red by-products of high retention on the reversed phase are also formed during this reaction step. However, these differ slightly in their optical properties from the red compounds formed during the carboxymethylation and rearrangement (λ_{max} = 520 and 570 nm).

Identification of the peaks

In order to identify the compounds separated (*cf.* Figs. 2A, 3A and 4A), comparisons with standards (AMP, iodoacetic acid and iodide) and the tests described in the Methods were both performed. Measurements of UV absorption spectra revealed which nitrogen atom of the adenine ring had been substituted. AMP and I have the

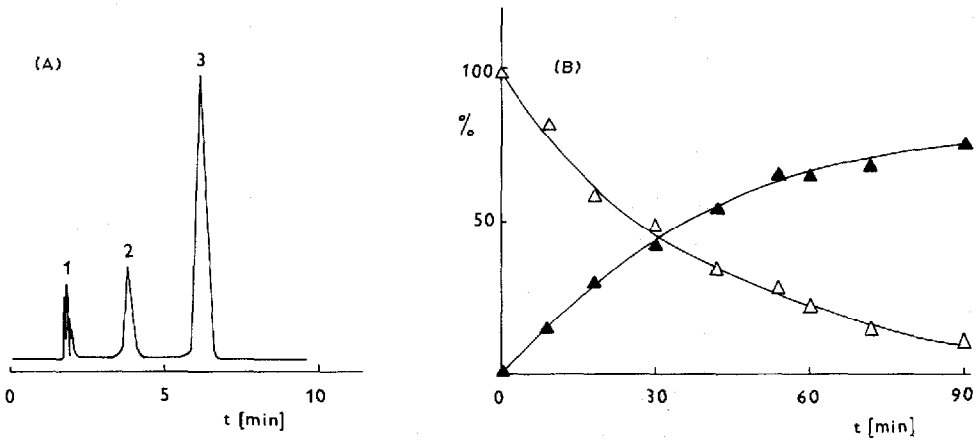

Fig. 4. Binding of diaminohexane to N^6-carboxymethyl-AMP. (A) Separation of the reaction mixture. Mobile phase, 10% methanol in buffer (pH 2.5); other conditions were the same as in Fig. 2. Peaks: 1 = unknowns; 2 = N^6-carboxymethyl-AMP (II); 3 = N^6-[N-(6-aminohexyl)carbamoylmethyl]-AMP (III). (B) Reaction course monitored by HPLC. △ = II; ▲ = III. Results are expressed as percentages of the initial concentration of II.

absorption maximum at 259 nm (ε_{259} of AMP = 15.4 mM^{-1} cm^{-1}) and compound I has an additional shoulder at 290 nm[7], whereas the compounds substituted at N^6 (II and III) are characterized by the absorption maximum at 267 nm (ε_{267} = 17.3 mM^{-1} cm^{-1})[7]. IR spectra helped to confirm the presence of carbonyl groups in I, II and III (typical bands between 1600 and 1700 cm^{-1}) and a carboxy group in I and II (characteristic broad bands at 2500–3000 cm^{-1}). The anodic peaks observed in the cyclic voltammograms of AMP, II and III (at −10 mV, with prepolarization of −1.75 V) agree with the presence of unsubstituted N^1 in these molecules[8]. Compound I, which is devoid of a free electron pair on N^1, does not show this anodic peak. The free amino group of III was identified by the reaction with ninhydrin; compound III gave violet products whereas the other compounds yielded only faint pink colours.

Dependence of retentions on pH

The capacity factors of the compounds under study are strongly affected by the pH value of the mobile phase (Fig. 5). The retention of AMP at first increases with growing pH (with the inflection point between pH 6 and 6.5). The ascending part of the curve can be explained by deprotonation of the N^1 atom of adenine, while the descending part might correspond to ionization of the phosphate group. The approximate inflection points are in good agreement with the pK values of AMP published in ref. 9 (pK_1 = 3.74, pK_2 = 6.05). In the case of compounds I and II the effect of phosphate ionization at *ca.* pH 6 can also be observed. In addition, compound II exhibits a steep decrease in the originally fairly high capacity factor between pH 4 and 5; this can be connected with the dissociation of its carboxy group. This effect is suppressed in the polar compound I, probably because of the influence of the positive charge of the N^1 atom on the carboxy group.

Modification of the purification procedure

HPLC provided an excellent method for monitoring the reaction couse during

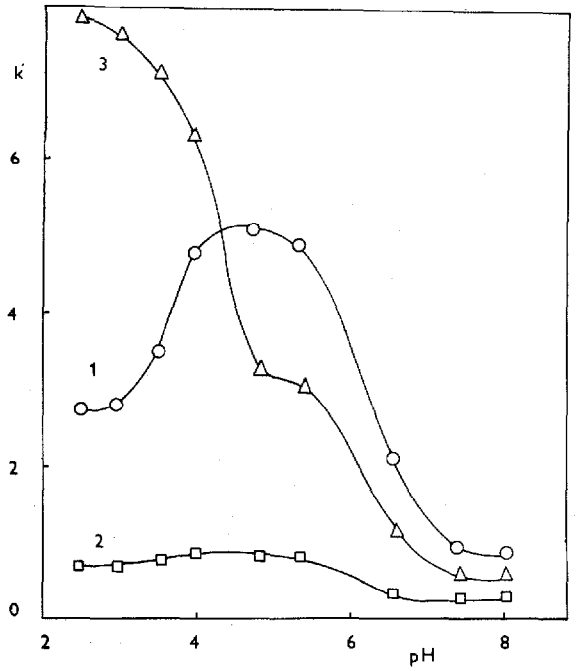

Fig. 5. Dependence of capacity factors (k') on the pH of the mobile phase. Conditions as in Fig. 2A. Curves: 1 = AMP; 2 = N^1-carboxymethyl-AMP; 3 = N^6-carboxymethyl-AMP.

all steps of the synthesis of III and during all the purification procedures used. As shown in Figs. 2B, 3B and 4B, the conditions described in ref. 5 for the chemical reactions are almost optimal. No changes of reaction conditions (*i.e.* pH, temperature, concentration of reagents and incubation time) led to significant improvements of yields or to any reduction of the amounts of by-products. The only exception was prolonging the coupling time during the reaction of diaminohexane with II from 60 to 90 min (*cf.* Fig. 4B). If carbodiimide is added more quickly than in our case (dropwise addition for the first 50 min of the reaction), the reaction time can be reduced to that recommended in ref. 5 when only a slightly lower yield of III is attained. However, in this case the amount of undesirable by-products is increased.

On the other hand, various simplifications of the original purification steps performed between the consecutive chemical reactions can be proposed on the basis of the HPLC analysis. The original purification method[5] requires two separations on large columns packed with Dowex 1-X2 (30 × 4 cm) using time-consuming elutions of products with gradients of lithium chloride solutions. These chromatographic separations are performed after the rearrangement and after the reaction with diaminohexane.

HPLC analysis enabled us to propose two variations in the separation procedure. In the first variant, we used three small columns with Dowex 1-X2 after each chemical reaction (*cf.* Fig. 1). These purification steps led to the separation of coloured by-products and of other undesirable compounds. The synthesis was performed with 700 mg (2 mmoles) of AMP. After the carboxymethylation the product was precipitated using ethanol[5] dissolved in a small amount of 0.2 *M* lithium chloride (pH

6) and loaded onto the Dowex column (5 × 1 cm) equilibrated with the same solution. The column was washed with 0.2 *M* lithium chloride (pH 6) until HPLC analysis indicated the end of the elution of I (*ca.* 20 ml eluate). In this way, compound I can be obtained almost pure. Because the rearrangement could be performed with purified I, only a small amount of coloured by-products was formed. The volume of the reaction mixture after rearrangement was reduced by evaporation to *ca.* 5 ml, the pH was brought to 6.0 and the solution was applied to a similar column packed with Dowex as described above. The elution was monitored by HPLC and pure II was collected and used for the reaction with diaminohexane. The product was then precipitated with ethanol[5], dissolved in a small volume of 0.05 *M* lithium chloride (pH 5) and loaded onto the column containing Dowex 1-X2 (7 × 1.5 cm) and equilibrated with the same solution. The column was washed with the same solution until the eluate was devoid of impurities (HPLC). The product III was eluted with 0.2 *M* lithium chloride (pH 3), the solution was neutralized and concentrated and a precipitation with cold ethanol–acetone (1:1) was carried out. Approximately 240 mg of almost pure III was obtained.

The high retention of compound III on the reversed phase can be regarded as the basis for another simple variant of the purification procedure. In this case 70 mg (0.2 mmoles) of AMP was used in the experiment. All the chemical reactions described in the Methods (*cf.* Fig. 1) were carried out without any purification of the intermediates. The crude final product III was purified by passage through a small column of Dowex 1-X2 and by chromatography on the reversed-phase. The red by-products which could cause the separation properties of the reversed phase to deteriorate were completely retained on a small Dowex column (2 × 1 cm) equilibrated with 0.05 *M* lithium chloride (pH 5). The product III (together with the intermediates and other compounds) was eluted with 0.2 *M* lithium chloride (pH 3). The eluate was then applied to the semi-preparative HPLC column containing the reversed phase. The column was washed with 25 m*M* phosphate buffer (pH 2.5) until the UV absorbance dropped. Thereafter, consecutive elutions with 4, 10 and 50% solutions of methanol in the buffer were performed. Almost pure III (*ca.* 20 mg) was eluted with 10% methanol.

The combination of both proposed separation procedures (*i.e.* passage through small columns containing Dowex 1-X2 after each chemical reaction accompanied by semi-preparative HPLC) gave the best results regarding purity of the final product III.

REFERENCES

1 K. Mosbach, *Methods Enzymol.*, 34 (1974) 229.
2 M. Lindberg and K. Mosbach, *Eur. J. Biochem.*, 53 (1975) 481.
3 M. Zakaria and P. R. Brown, *J. Chromatogr.*, 226 (1981) 267.
4 V. Kahle, J. Kovář, K. Šimek and K. Tesařík, *Anal. Biochem.*, 115 (1981) 245.
5 K. Mosbach, P. O. Larsson and Ch. Lowe, *Methods Enzymol.*, 44 (1976) 859.
6 V. Kahle and K. Tesařík, *Chem. Listy*, 74 (1980) 874.
7 M. Lindberg, P. O. Larsson and K. Mosbach, *Eur. J. Biochem.*, 40 (1973) 187.
8 H. Klukanová, *Thesis*, Purkynje University, Brno, 1982.
9 H. M. Rauen, *Biochemisches Taschenbuch*, Springer, Berlin, 1964, Vol. 2, p. 81.