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LIQUID CHROMATOGRAPHIC SEPARATIONS OF FLUORESCENT 1,N^6-ETHENOADENOSINE DERIVATIVES OF AMP, ATP AND NAD

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SUMMARY

High-performance reversed-phase (C_{18}) and anion-exchange chromatography of the title compounds is reported. Stationary phases of both types proved to be very convenient for an efficient analysis of the preparations of these compounds. Anion-exchange columns with quaternary amines (Pharmacia Mono Q columns) and those with tertiary amines (DEAE-Spheron columns) yielded comparable results. Identification of the peaks was performed by means of ultraviolet and fluorescence spectroscopic measurements. Microgram to miligram quantities of the title substances can be separated in less than 20 min using analytical columns. For preparative purposes, up to 30 mg of these compounds can be separated on larger DEAE-Spheron columns (120 × 10 mm I.D.) in a single injection. Analysis of the reaction mixtures [containing oxidized nicotinamide-adenine dinucleotide (NAD) and variable amounts of chloroacetaldehyde under different conditions] enabled us to modify the reaction and separation conditions to increase the yield of 1,N^6-etheno-NAD.

INTRODUCTION

The treatment of adenosine nucleotides [*e.g.* AMP, ATP and oxidized nicotinamide-adenine dinucleotide (NAD)] with chloroacetaldehyde in aqueous solutions at mild pH and temperatures yields the corresponding fluorescent 1,N^6-ethenoadenosine derivatives of these compounds (*e.g.* ε-AMP, ε-ATP and ε-NAD)[1–3]. These derivatives can replace AMP, ATP and NAD in their specific interactions with kinases and dehydrogenases and provide very suitable fluorescence probes for studying the kinetic and binding properties of these enzymes[1–5]. The preparations of these substances are relatively easy[1–3]; ε-AMP and ε-ATP are usually obtained in high yields as nearly pure compounds[1], the yield and purity of ε-NAD use to be rather lower[3]. The course of the syntheses of these compounds has been followed by thin-layer chromatography (TLC) techniques[1–3] and chromatography on Dowex (1 × 1) columns was used as an essential purification step in the preparation of ε-NAD[3]. The

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progress in high-performance liquid chromatography (HPLC) in the last decade showed that the separations on reversed phases and anion-exchange high-performance materials are the most efficient methods for the analysis and preparation of adenosine nucleotides and their derivatives[6–9].

The aim of this paper is to show the possibility of monitoring the synthesis of ε-AMP, ε-ATP and ε-NAD by HPLC on C_{18} reversed phases and anion exchangers (quaternary and tertiary amines), to demonstrate the contribution of an exact HPLC analysis to the improvement of the reaction conditions in the syntheses of these compounds and to present the results of a preparative HPLC separation of ε-NAD.

EXPERIMENTAL

Materials

AMP and ATP were obtained from Reanal (Budapest, Hungary), and NAD was obtained from Imuna (Šarišské Michalany, Czechoslovakia). These compounds were found to contain < 5% impurities by reversed-phase HPLC (see below) and were used without further purification. Chloroacetaldehyde (*ca.* 50% solution in water) was obtained from Fluka (Buchs, Switzerland). Pyrazole was purchased from Aldrich (Milwaukee, WI, U.S.A.), and tris(hydroxyethyl) aminomethane (Tris) was obtained from Merck (Darmstadt, F.R.G.). Horse liver alcohol dehydrogenase was purified as described[10], its purity being about 90%. Sephadex G-10 was purchased from Pharmacia (Uppsala, Sweden). Diethylaminoethyl-Spheron (DEAE-Spheron) (12 or 20 μm) and the other chemicals were obtained from Lachema (Brno, Czechoslovakia).

Synthesis of ε-AMP and ε-ATP

These substances were prepared essentially according to Secrist *et al.*[1]. A solution of 1 mmol of AMP or ATP in 20 ml of 1 *M* aqueous chloroacetaldehyde at about pH 4.5 (additions of lithium hydroxide) was stirred at room temperature for 2–3 days, until the HPLC analysis (see below) indicated that the amount of the parent compound dropped below 5% and until the absorbance ratio (A_{265}/A_{275}) became constant. The product was decolourized with charcoal and lyophilized. The derivatives were recrystallized from cold ethanol (0°C). The yields of ε-AMP and ε-ATP were about 90% (see below and *cf.* ref. 1). The purified substances were stored at −60°C.

Synthesis of ε-NAD

The methods of Barrio *et al.*[2] and Luisi *et al.*[3] were slightly modified (see Results and Discussion). A solution of 0.5 mmol of NAD in 15 ml of 1 *M* aqueous chloroacetaldehyde at pH 5.5–6.0 (additions of lithium hydroxide) was incubated at 35°C for 24 h (until the reaction mixture contained < 5% NAD, see below). The product was decolourized with charcoal and precipitated with cold ethanol (0°C). The precipitate was dissolved in a small amount of water (pH 8.3) and applied to a column (120 × 10 mm I.D.) packed with DEAE-Spheron (20 μm). This column was attached to the chromatographic system described below. The separation of ε-NAD from NAD proceeded isocratically (10 m*M* Tris–HCl buffer, pH 8.3) and the impurities were eluted with a sodium chloride concentration gradient (see below). The

crude material was chromatographed in seven repeated runs, the yield being *ca.* 70% of the injected amount of ε-NAD. The nearly pure ε-NAD was desalted on a Sephadex G-10 column (600 × 7.5 mm I.D.), concentrated by evaporation and re-chromatographed on a Mono Q column (Pharmacia, Uppsala, Sweden), see below. The yield of ε-NAD was *ca.* 35%. The desalted and lyophilized ε-NAD was stored at −60°C.

HPLC separations

The chromatographic system used for the separations on reversed-phases consisted of a VCM-300 pump (Laboratory Instruments, Prague, Czechoslovakia), a glass column (150 × 4 mm I.D.) packed with Separon C_{18} (5 μm) (Laboratory Instruments), a variable-wavelength UV monitor (Knauer, Berlin, G.F.R.) (λ = 265 nm) and/or a RF-530 fluorescence detector (Shimadzu, Kyoto, Japan). The chromatograms were recorded by means of a two-channel recorder (Knauer). The samples were injected with a syringe sample injector with a 10-μl sample loop (Rheodyne, Cotati, CA, U.S.A.); a FRAC-100 collector (Pharmacia) was used for collection of the peaks. This equipment was also used in preliminary experiments with a glass column (150 × 4 mm I.D.) containing Separon-amine (5 μm) (Laboratory Instruments).

Anion-exchange chromatography was performed using a Mono Q HR 5/5 column (50 × 5 mm I.D.) (Pharmacia) or a home-made glass column (50 × 5 mm I.D.) packed with DEAE-Spheron (12 μm). These columns were attached to a fast protein liquid chromatography (FPLC) system (Pharmacia) consisting of two P-500 pumps, a G-250 gradient programmer, a UV-1 monitor (λ = 254 nm), a REC-482 recorder and a FRAC-100 collector. The samples were injected by means of a V-7 valve (Pharmacia) with a 25-μl sample loop. A 500-μl loop was used in preparative separations.

Identification of the compounds

The ultraviolet spectra of the products were scanned with a Cary 118 (Varian, Palo Alto, CA, U.S.A.); the fluorescence emission spectra were measured with a standard Aminco-Bowman spectrophotofluorimeter (SLM/Aminco, Urbane, IL, U.S.A.). The ultraviolet spectra of 1,N⁶-adenosine derivatives are characterized with three absorption maxima at about 255, 265 and 275 nm (at pH 4–9) and a shoulder at *ca.* 310 nm (at pH > 5), *cf.* refs. 1–3. The concentrations of the compounds were determined spectrophotometrically using the published[1–3] values of absorption coefficients at 265 nm. ε-AMP and ε-ATP fluoresce strongly at *ca.* 410 nm (λ_{exc} = 310 nm)[1]. ε-NAD was identified fluorimetrically in the presence of 10 μ*M* horse liver alcohol dehydrogenase and 1.5 m*M* pyrazole in 0.1 *M* sodium phosphate buffer (pH 7) at 25°C (essentially according to ref. 3). ε-NAD fluoresces slightly (λ_{exc} = 310 nm, λ_{em} = 410 nm); the fluorescence intensity is nearly 15-fold in the presence of alcohol dehydrogenase and pyrazole.

RESULTS AND DISCUSSSION

HPLC separations of ε-AMP and ε-ATP

High-performance liquid chromatography on C_{18} reversed-phase or strong anion exchangers proved to be very convenient for the analysis of the products

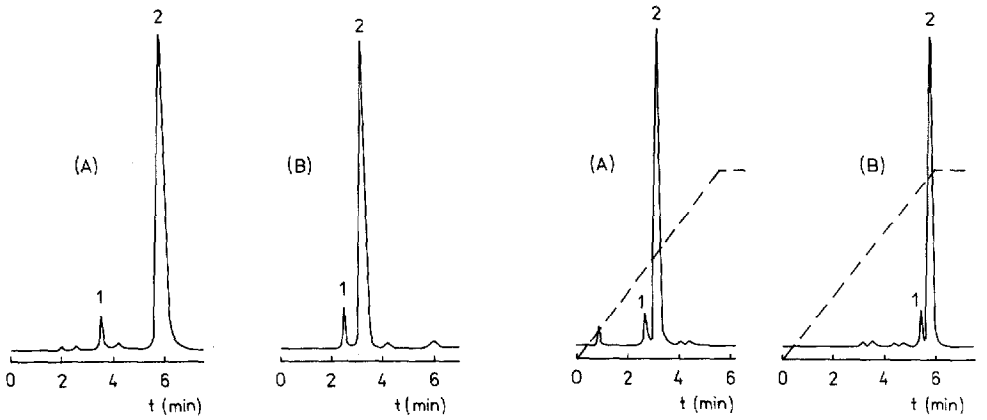
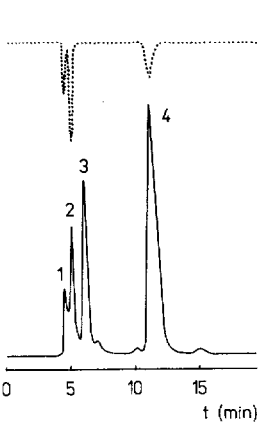


Fig. 1. Reversed-phase HPLC separation of (A) ε-AMP and (B) ε-ATP. Column, Separon C_{18}; mobile phase, 30 m*M* sodium phosphate buffer (pH 3); flow-rate, 0.5 ml/min; λ = 265 nm. Peaks 1 were AMP (A) and ATP (B); peaks 2 had the properties of ε-AMP (A) and ε-ATP (B) (see Experimental).

Fig. 2. Anion-exchange HPLC separation of (A) ε-AMP and (B) ε-ATP. Column, Mono Q HR 5/5; gradient elution (----), 10 m*M* Tris–HCl buffer (pH 8.3) (solution A in both instances), the same buffer with 0.3 *M* sodium chloride (solution B in both instances); flow-rate, 1 ml/min; λ = 254 nm. Peaks 1 were AMP (A) and ATP (B); peaks 2 had the properties of ε-AMP (A) and ε-ATP (B).

formed in the reactions of chloroacetaldehyde with AMP and ATP (Figs. 1 and 2). The reversed-phase HPLC performed isocratically was found sufficient for the separations of $1,N^6$-ethenoadenosine derivatives from the parent compounds as well as from the impurities; these separations were completed within 6 min (Fig. 1). The best separation of the derivatives from the parent compounds was attained at pH 3. Both unsubstituted and substituted adenine rings are protonated at this pH value (their p*K* values are in the range of 3.8–4.3, *cf.* refs. 1 and 11) and the differences in their hydrophobic interactions with the stationary phase might be decisive for the separation of the corresponding substances. Accordingly, the retentions of ε-AMP and ε-ATP were higher in comparison with those of the parent compounds at this pH (Fig. 1). The retentions of the derivatives in separations on anion-exchangers (at pH 8.3 using gradients of sodium chloride concentrations) were also higher than those of the initial compounds (Fig. 2). The peaks identified as ε-AMP and ε-ATP had the properties of $1,N^6$-ethenoadenosine derivatives; they exhibited characteristic UV spectra at pH 7 (see Experimental) and fluoresced strongly (λ_{exc} = 310 nm, λ_{em} = 410 nm). The peaks identified as AMP and ATP in Figs. 1 and 2 had retention times identical to the parent compounds, they had simple absorption peaks at *ca.* 260 nm and did not fluoresce. The results shown in Figs. 1 and 2 indicate that the conversion of AMP and ATP to the corresponding $1,N^6$-ethenoadenosine derivatives is nearly complete (the yields of the syntheses being *ca.* 90%, *cf.* ref. 1) and that there is no reason to change the reaction conditions described[1]. The HPLC analysis provides a quick and efficient method for the determination of the ε-AMP and ε-ATP content in the obtained preparations and a suitable method for monitoring the course of the synthesis. Moreover, the described chromatography on a Mono Q column (Fig. 2) can be used for semi-preparative separations of several miligrams of ε-AMP and ε-



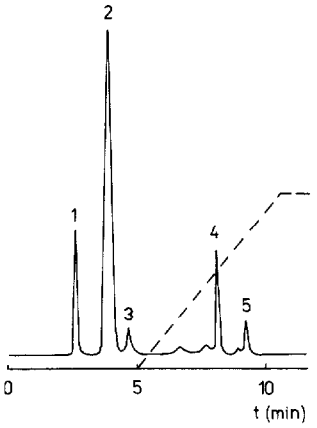


Fig. 3. Reversed-phase HPLC separation of ε-NAD. The mixture of 33 m*M* NAD and 1 *M* chloroacetaldehyde incubated for 11 h at 35°C was analysed. Column, Separon C_{18}; mobile phase, 30 m*M* sodium phosphate buffer (pH 5.5); flow-rate, 0.5 ml/min; absorbance at 265 nm (——); fluorescence at λ_{exc} = 310 nm, λ_{em} = 410 nm (.....). Peaks: 1 = ε-ADP and unknown; 2 = ε-AMP; 3 = NAD; 4 = ε-NAD.

Fig. 4. Anion-exchange HPLC separation of ε-NAD. The same conditions as given in Fig. 2 were used and the same sample as in Fig. 3 was analysed. Peaks: 1 = NAD, 2 = ε-NAD, 3 = unknown (λ_{max} = 265, 360 nm), 4 = ε-AMP, 5 = ε-ADP.

ATP. The chromatograms obtained with a column containing DEAE-Spheron were similar to those shown in Fig. 2, the resolutions being slightly worse. The use of larger columns (120 × 10 mm I.D.) packed with DEAE-Spheron (20 μm) can yield up to 30 mg of pure ε-AMP or ε-ATP in a single run. The chromatographically purified 1,N⁶-ethenoadenosine derivatives of AMP and ATP can be used for the most exact enzymologic experiments.

HPLC separations of ε-NAD

The chromatograms of the reaction mixtures containing NAD and chloroacetaldehyde obtained with a C_{18} reversed-phase showed that the formation of undesirable by-products was more significant than in the case of ε-AMP and ε-ATP synthesis (*cf.* Figs. 1 and 3). These impurities were eluted prior to NAD (peaks 1 and 2 in Fig. 3). The main constituent of peak 1 might be ε-ADP; peak 2 might be identical with ε-AMP (see below). The separation of ε-NAD from NAD by reversed-phase HPLC was good with sodium phosphate buffer (pH 5.5); the isocratic elution was sufficient for a satisfactory resolution of the peaks. The results of anion-exchange HPLC on a Mono Q column (Fig. 4) confirmed those obtained with reversed-phase HPLC; NAD and ε-NAD separated well using isocratic elution with 10 m*M* Tris–HCl buffer (pH 8.3). The unknown compound eluted behind ε-NAD (peak 3 in Fig. 4), with absorption maxima at about 265 and 360 nm; it might be responsible for the yellow colour of the concentrated solutions of crude ε-NAD. Further impurities were eluted with increasing sodium chloride concentration in the mobile phase; the most important impurity could correspond to ε-AMP. The UV spectra and fluorescence properties of peak 2 in Fig. 3 and of peak 4 in Fig. 4 were identical with those of ε-AMP (see Experimental); this compound was eluted from a Mono Q column at the

same sodium chloride concentration as ε-AMP in Fig. 2. It is probable that peak 5 in Fig. 4 is identical with the main constituent of peak 1 in Fig. 3; it might correspond to ε-ADP (due to its optical properties and its positions in the chromatograms). The elution profile obtained with a DEAE-Spheron column of identical dimensions using the same elution as in the case of a Mono Q column was nearly the same as that shown in Fig. 4. Preliminary experiments with a column packed with Separon-amine (containing NH_2 groups) performed at pH 5–6 yielded essentially similar results as those carried out with the columns containing stationary phases with quaternary or tertiary amines.

Modified preparation of ε-NAD

The reaction conditions recommended[2,3] for the synthesis and purification of ε-NAD led to the formation of not completely pure ε-NAD (as shown by HPLC chromatography; see the conditions described in Figs. 3 and 4), the yield being lower than 20%. Reversed-phase HPLC provided a convenient method for monitoring the reaction course during the synthesis and during the purification steps. The conditions were changed and the results were evaluated on the basis of the HPLC analysis. A concentration of chloroacetaldehyde of 1 *M* was found suitable for a sufficiently high reaction rate; higher concentrations of this reagent brought about an increased formation of degradation products (peaks 1 and 2 in Fig. 3). A temperature of 35°C was found to be the optimum; the reaction rate decreased to about one half at 20°C. On the other hand, temperatures above 40°C resulted in more contaminated reaction products. The pH value of the reaction mixture was kept in the range of 5.5–6.0; these conditions gave better results than the recommended[2,3] lower pH values. The reaction was completed within 24 h under the optimum conditions (Fig. 5). The amount of NAD dropped below 5% of the initial value; ε-NAD constituted nearly 80% of the reaction product and the amount of impurities (peaks 1 and 2 in Fig. 3) did not exceed 20%.

The purification procedure was also modified on the basis of the HPLC results. The decolorization of the crude reaction product with charcoal[3] was necessary to remove the yellow impurity (it might correspond to peak 3 in Fig. 4, see above); the precipitation with cold ethanol was a suitable step removing the non-nucleotide components of the reaction mixture (see Experimental). Preparative HPLC separation on

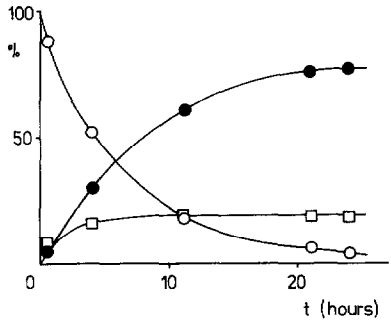


Fig. 5. Synthesis of ε-NAD analysed by reversed-phase HPLC. The reaction conditions are given in the Experimental; ● = ε-NAD, ○ = NAD, □ = impurities (corresponding to peaks 1 and 2 in Fig. 3).

a DEAE-Spheron column was carried out under similar conditions as described in Fig. 4. The separation of ε-NAD from NAD and most of the impurities (peaks 4 and 5 in Fig. 4) was good, whereas a small amount of the unknown (λ_{max} = 265, 360 nm; peak 3 in Fig. 4) remained in the ε-NAD preparation. This impurity was removed completely if the desalted product was re-chromatographed on a Mono Q column (see Experimental).

The ε-NAD obtained was found to be homogeneous by reversed-phase HPLC, its optical properties being identical with those described[3]. Free ε-NAD exhibited a moderate fluorescence at about 410 nm, its fluorescence intensity being essentially lower than that of ε-AMP (*cf.* also Fig. 3). This is due to the intramolecular quenching of the nucleotide fluorescence caused by the stacked conformation of its molecules in water solutions[3]. The fluorescence intensity increased essentially (and its emission maximum shifted to *ca.* 405 nm) upon addition of alcohol dehydrogenase and pyrazole (see ref. 3 and Experimental). This specific test based on the formation of the specific ternary complex of ε-NAD with the enzyme and pyrazole (accompanied with the opening of the stacked ε-NAD conformation[3]) proved the full competence of the prepared ε-NAD as a fluorescence probe for NAD-dependent dehydrogenases.

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