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Capillary electrophoresis of nicotinamide–adenine dinucleotide and nicotinamide–adenine dinucleotide phosphate derivatives in coated tubular columns

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Abstract

HPCE was shown to be an effective and convenient method for the determination of nicotinamide–adenine dinucleotide (oxidized, NAD^+; reduced, NADH), nicotinamide–adenine dinucleotide phosphate (oxidized, NADP; reduced, NADPH) and their synthetic derivatives. The coenzymes were easily separated among themselves and from their degradation products, which are inhibitors of dehydrogenases, in 15 min in a coated capillary. Several coenzyme derivatives such as N^6-(2-aminoethyl)-$NAD(P)^+$ and N(1)-(2-aminoethyl)-$NAD(P)^+$ were separated by zone electrophoresis in uncoated or coated capillaries using 50 m*M* 3-(N-morpholino)propanesulphonic acid (pH 7.0) or Tris–HCl (pH 8.0) as buffer systems. Capillary zone electrophoresis and micellar electrokinetic capillary chromatography can also be used to monitor continuously coenzyme chemical modifications.

1. Introduction[☆]

The pyridine coenzymes play a central role in the metabolic activities of plants, animals and microorganisms. The availability of NAD^+, $NADP^+$, NADH and NADPH to investigators has improved dramatically since the introduction of ion-exchange chromatography, which allowed the production of nearly pure NAD^+ in gram amounts. The Dowex chromatography developed by Kornberg [1] was the basis for the commercial preparation of NAD^+ from baker's yeast. $NADP^+$, first isolated from red blood cells and then from yeast, in turn obtained from NAD^+ through the NAD-kinase [2] catalysed phosphorylation.

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☆ Abbreviations: NAD^+ = nicotinamide–adenine dinucleotide, oxidized; NADH = nicotinamide–adenine dinucleotide, reduced; $NADP^+$ = nicotinamide–adenine dinucleotide phosphate, oxidized; NADPH = nicotinamide–adenine dinucleotide phosphate, reduced.

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The chemical stability of pyridine coenzymes has been the subject of extensive investigations since the coenzymes were first isolated [3]. Their reactivity towards acidic or basic pH is remarkably complementary, with both oxidized and reduced coenzymes fortuitously stable in a narrow range of pH, centred on physiological conditions.

A considerable number of $NAD(P)^+$ analogues with simple or multiple structural alterations have been synthesized for a variety of experimental needs [4–11]. Analogues have been prepared containing fluorescent bases, reactive groups for covalent modifications of enzymes or, more generally, derivatives designed to investigate structure–function relationships. During the last 15 years, interest has focused on the development of methods for synthesizing macromolecular NAD(H) and NADP(H) derivatives to be used in continuous-flow membrane reactors [12]. Modification of coenzymes outside the active centre generally does not cause a marked loss of enzymatic activity and the analogues can bind to the enzyme and participate in the hydrogen transfer similarly to native coenzymes [13].

Chromatographic methods have been widely applied for the determination of the purity of the pyridine coenzymes and for the determination of the analogues. These methods include chromatography on DEAE-cellulose [14], DEAE-Sephadex [15,16], benzoylated DEAE-cellulose [17], AG MP-1 [18] and reversed-phase high-performance liquid chromatography [19,20].

Capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) appear to be attractive tools for the determination of pyridine coenzymes, because of their high separation efficiency, easy operation and low running costs.

In this paper we describe the separation of NAD^+, NADH, $NADP^+$ and NADPH among themselves and from their degradation products and analogues by CZE and MECC. Separations were obtained by CZE in coated or uncoated capillaries according to the electrophoretic mobility of the compounds, or by MECC on the basis of their hydrophobicity or ionic character.

2. Experimental

2.1. Materials

Nicotinamide–adenine dinucleotide (NAD^+), nicotinamide–adenine dinucleotide reduced form (NADH), nicotinamide–adenine dinucleotide phosphate ($NADP^+$), nicotinamide–adenine dinucleotide phosphate reduced form (NADPH), adenosine 5′-diphosphoribose (ADPR), adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), 3-(N-morpholino)propanesulphonic acid (MOPS), sodium dodecyl sulphate (SDS) and Tris were purchased from Sigma (St. Louis, MO, USA). N-Acryloylaminoethoxyethanol was synthesized as described by Chiari *et al.* [21]. Ammonium peroxodisulphate and N,N,N′,N′-tetramethylenediamine (TEMED) were obtained from Bio-Rad Labs. (Richmond, CA, USA). N(1)-Carboxymethyl-NAD^+, N^6-(2-aminoethyl)-NAD^+, N(1)-(2-aminoethyl)-NADP, N^6-(2-aminoethyl)-$NADP^+$ and N^6-(2-hydroxy-3-trimethylammoniumpropyl)-NAD^+ were synthesized as already described [12].

2.2. Methods

CZE was performed in a Waters Quanta 4000 capillary electrophoresis system (Millipore, Milford, MA, USA). For the experiments, uncoated and coated (modified Hjertén procedure [22]) fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) 40 cm (35 cm to the detector) long were used. The separations in uncoated capillaries were carried out using 50 m*M* Tris–HCl buffer (pH 8.0) or 50 m*M* MOPS buffer (pH 7.0) in the presence of 50 m*M* SDS and 10% methanol. The separations in coated capillaries were performed in 50 m*M* MOPS buffer (pH 7.0). The samples were loaded by hydrostatic pressure and the separations were carried out at room temperature. The detector was set at 254 nm.

2.3. Coating the capillary wall

The following procedure gave the best results. The capillary was first treated with 100 μl of 1 *M*

NaOH for 5 h, then rinsed and flushed with 100 μl of 0.1 *M* HCl followed by 100 μl of 0.1 *M* NaOH. After 1 h it was rinsed with water and acetone, filled with a 1:1 solution of Bind Silane [3-(trimethoxysilyl)propyl methacrylate] in acetone and then incubated overnight. After this treatment, the capillary was flushed with air for 5 min and then washed with 20 m*M* phosphate buffer (pH 7.0). The capillary was filled with 6% N-acryloylaminoethoxyethanol solution in the same degassed buffer containing the appropriate amount of catalyst (0.5 μl of TEMED and 0.5 μl of 40% ammonium peroxodisulphate per ml of gelling solution). Polymerization was allowed to proceed overnight at room temperature and then the capillary was emptied by means of a syringe.

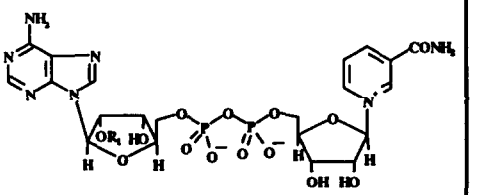
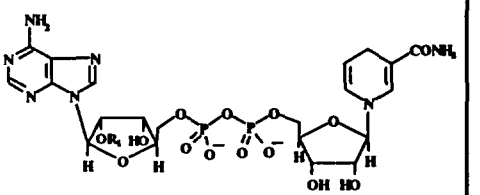
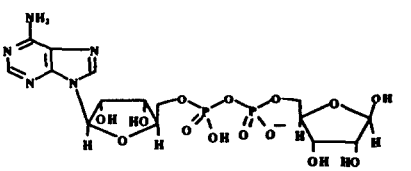
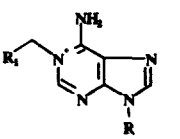
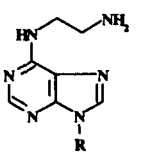
NAD^+ $R_1 = H$ (1) $NADP^+$ $R_1 = PO_3^=$ (2)	
NADH $R_1 = H$ (3) NADPH $R_1 = PO_3^=$ (4)	
Adenosine 5'-diphosphoribose (5)	
N(1)-(2-aminoethyl)-NAD^+/$NADP^+$ $R_1 = CH_2NH_2$ (6) N(1)-carboxymethyl-NAD^+ $R_1 = COOH$ (7)	
N^6-(2-aminoethyl)-NAD^+/$NADP^+$ (8)	

Fig. 1. Structures of $NAD(P)^+$/NAD(P)H derivatives.

3. Results and discussion

The structures of the coenzymes and of their degradation products and analogues are shown in Fig. 1. A mixture of NAD^+, $NADP^+$, NADH, NADPH, AMP, ADP and adenosine 5′-diphosphoribose was resolved into seven well separated peaks by HPCE (Fig. 2). Identification was achieved by injecting either the mixture of products or a sample of each component. The CE separation reported was performed using different buffers in a coated capillary: 50 m*M* Tris–HCl (pH 8.0) or 50 m*M* MOPS (pH 7.0). Satisfactory resolution was achieved in both instances but MOPS is preferable as it allows a high voltage to be used as its conductivity is lower. The use of a coated capillary is essential in order to obtain sharp peaks and reproducible results.

Fig. 3A and B depict the electropherograms of

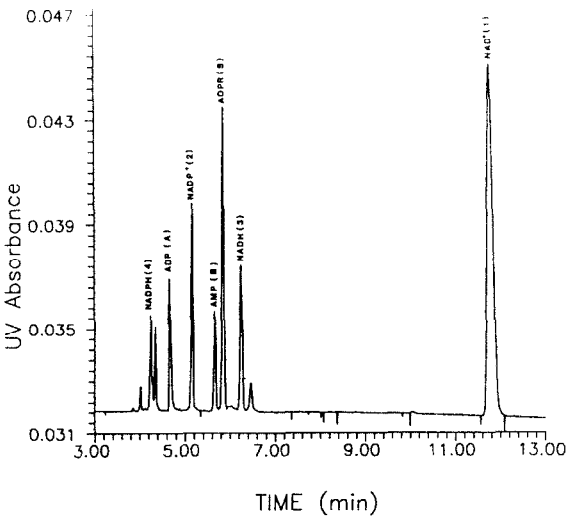


Fig. 2. CZE profile of a mixture of seven $NAD(P)^+$ and NAD(P)H derivatives. Conditions: Waters Quanta 4000 unit, fitted with a coated capillary of 45 cm × 100 μm I.D. with 50 m*M* MOPS (pH 7.0), run at 15 kV and 39 μA; sample concentration, 0.1 m*M* in 50 m*M* MOPS buffer; sample injection, 5 s by hydrostatic pressure; detection at 254 nm; anodic migration (reverse polarity). Peaks: 4 = nicotinamide–adenine dinucleotide phosphate ($NADP^+$), A = adenosine 5′-diphosphate (ADP); 2 = nicotinamide–adenine dinucleotide phosphate ($NADP^+$); B = adenosine 5′-monophosphate (AMP); 5 = adenosine 5′-diphosphoribose (ADPR); 3 = nicotinamide–adenine dinucleotide reduced form (NADH); 1 = nicotinamide–adenine dinucleotide (NAD^+).

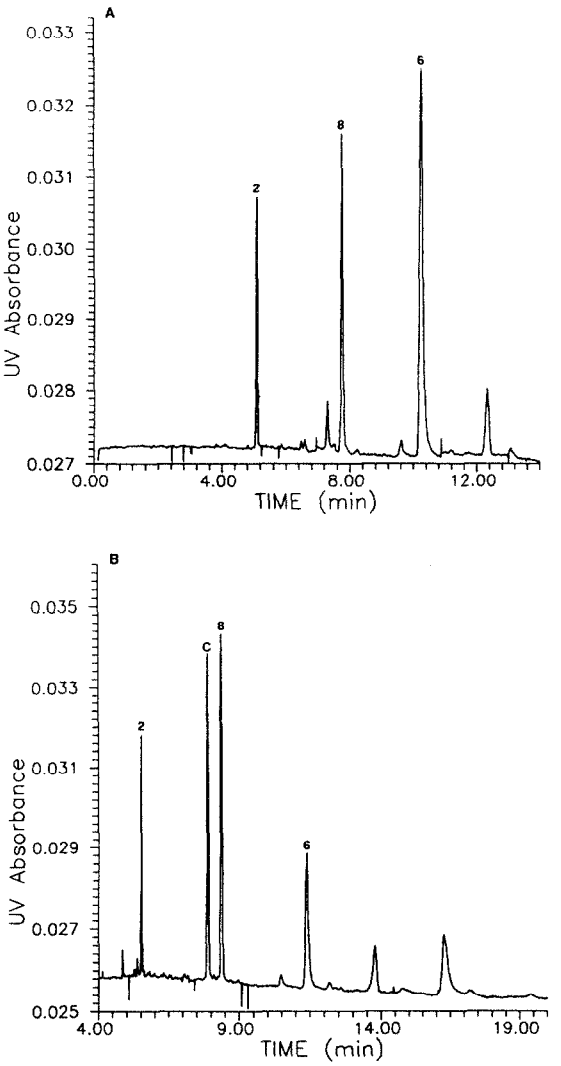


Fig. 3. (A) CZE separation of a mixture of $NADP^+$ derivatives. Conditions as in Fig. 1. Sample concentration, 0.03 mg/ml in 50 m*M* MOPS buffer; sample injection, 5 s by hydrostatic pressure; detection at 254 nm. Peaks: 2 = $NADP^+$; 8 = N^6-(2-aminoethyl)-$NADP^+$; 6 = N(1)-(2-aminoethyl)-$NADP^+$. (B) Analysis of Dimroth rearrangement products. Conditions as in Fig. 1. Sample concentration, 0.03 mg/ml in 50 m*M* MOPS buffer; sample injection, 5 s by hydrostatic pressure. Peaks: 2 = $NADP^+$; C = tricyclic 1,N^6-ethanoadenine-$NADP^+$; 8 = N^6-(2-aminoethyl)-$NADP^+$; 6 = N(1)-(2-aminoethyl)-$NADP^+$.

some $NADP^+$ derivatives. It can be seen that $NADP^+$ was easily and rapidly separated from N(1)-(2-aminoethyl)-$NADP^+$ and from N^6-(2-aminoethyl)-$NADP^+$ (Fig. 3A). The incubation of N(1)-(2-aminoethyl)-$NADP^+$ at pH 6.0–6.5 and 40–50°C yields, through a Dimroth rearrangement, N^6-(2-aminoethyl)-$NADP^+$ [12], which is the coenzymatically active form to be used for the covalent linking to soluble polymers [12,13]. In the rearrangement a low conversion of the N(1)- to the N^6-derivative occurs, as a parallel transformation to the tricyclic 1,N^6-ethanoadenine-$NADP^+$ takes place. Fig. 3B shows that the separation of the three reaction components is fast and complete, which facilitates the continuous monitoring of the reaction course. The detection limit for the coenzyme assay (at a signal-to-noise ratio of 3) was 2.0 μM and the response was linear in the concentration range 0.0025–5 mM. The unidentified peaks are probably degradation and secondary products of the reaction.

In Fig. 4 it can be seen that NAD^+, N(1)-(2-aminoethyl)-NAD^+ and N^6-(2-aminoethyl)-NAD^+ are resolved in an uncoated capillary in 5 min using Tris–HCl as buffer. Analogous conditions have been used for resolving NAD^+ from N^6-(2-hydroxy-3-trimethylammoniumpropyl)-NAD^+, as shown in Fig. 5. The electropherogram in Fig. 6 depicts a MECC separation of NAD^+ from the N(1)-carboxymethyl derivative. The running conditions chosen were 50 mM MOPS (pH 7.0)–50 mM SDS–10% methanol in an uncoated capillary. With the experimental conditions used the N(1)-carboxymethyl derivative was found to elute after 4 min.

Both commercial and enzymatically generated preparations of pyridine nucleotides, in particular the reduced forms, contain impurities. Some of these, such as adenosine 5′-diphosphoribose, are inhibitors of a number of dehydrogenases. The speed and the resolving capability of HPCE provide a valid method for the analysis of $NAD(P)^+$ and NAD(P)H impurities which are clearly separated in a single run. Most of the currently used purification and analytical procedures rely on anion-exchange chromatography on DEAE columns. These methods suffer from

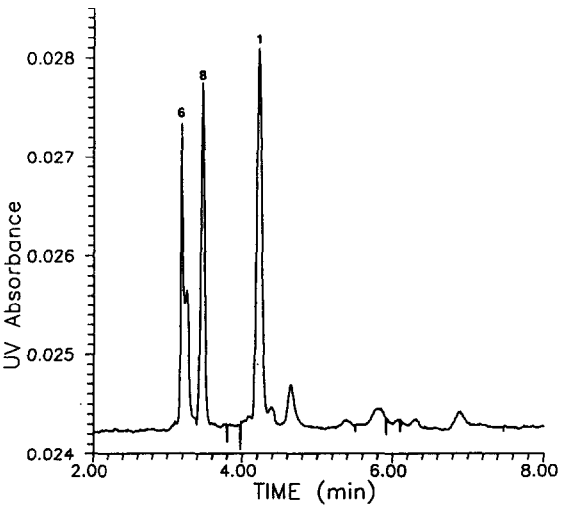


Fig. 4. CZE profile of a mixture of NAD^+ derivatives. Conditions: Waters Quanta 4000 unit, fitted with an uncoated capillary of 40 cm × 75 μm I.D. with 50 mM Tris–HCl (pH 8.0), run at 12 kV and 42 μA; sample concentration, 0.3 mg/ml in 50 mM Tris–HCl buffer; sample injection, 5 s by hydrostatic pressure; detection at 254 nm; cathodic migration. Peaks: 6 = N(1)-(2-aminoethyl)-NAD^+; 8 = N^6-(2-aminoethyl)-NAD^+; 1 = NAD^+.

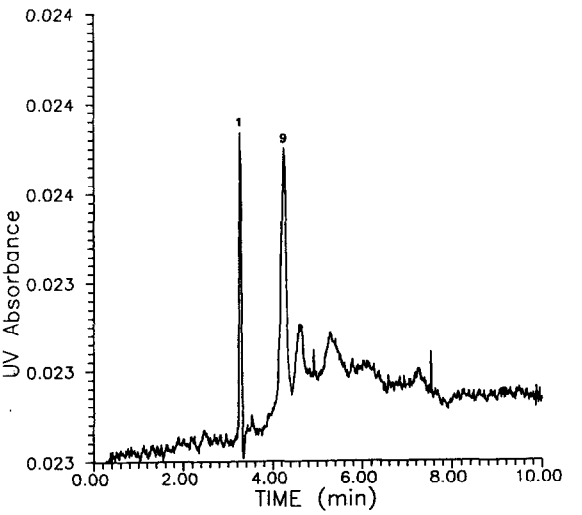


Fig. 5. CZE separation of NAD^+ (peak 1) from N^6-(2-hydroxy-3-trimethylammoniumpropyl)-NAD^+ (peak 9). Conditions as in Fig. 3. Sample concentration, 0.3 mg/ml in 50 mM Tris–HCl buffer; sample injection, 5 s by hydrostatic pressure; detection at 254 nm.

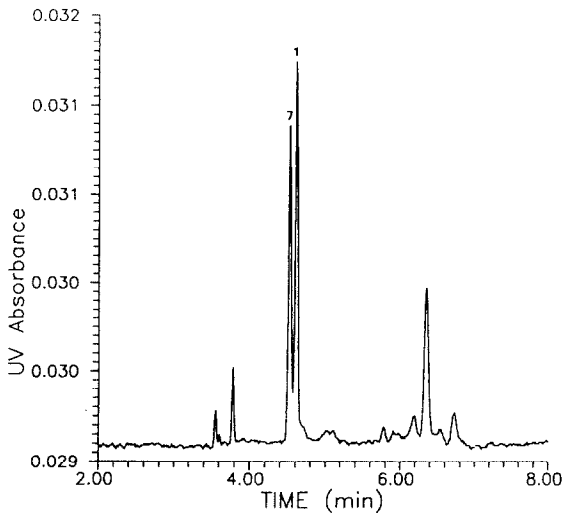


Fig. 6. MECC separation of N(1)-carboxymethyl-NAD$^+$ (peak 7) from NAD$^+$ (peak 1). Conditions: uncoated capillary of 40 cm × 75 μm I.D. with 50 m*M* MOPS (pH 7.0)–50 m*M* SDS–10% methanol, run at 12 kV and 35 μA; sample concentration, 0.15 m*M* in 50 m*M* MOPS buffer; sample injection, 5 s by hydrostatic pressure; detection at 254 nm; cathodic migration.

several disadvantages over that described here; for instance, NADH and NADP$^+$ are not resolved and ADPR is not separated from NADH [14–18]. Reversed-phase HPLC [18] easily separates NAD$^+$ from NADH but does not resolve very well NADP$^+$ from NADPH, which are eluted very close to the column void volume [23].

CE and MECC appear to be effective tools also for the determination of pyridine coenzyme derivatives. Some analogues are important probes for studying the structure–activity relationships and mapping the coenzyme binding sites of dehydrogenases, and others are used for various immobilization procedures, but in all instances a high level of purity of the synthetic derivatives is required for obtaining reproducible results. Among the low-molecular-mass precursors N^6-(2-aminoethyl)-NAD(P)$^+$ and N(1)-(2-aminoethyl)-NAD(P)$^+$ are of direct interest for use in dehydrogenase-based membrane reactors and for the preparation of affinity matrices.

In conclusion, the results described in this paper demonstrate the great effectiveness and convenience of HPCE for the determination of NAD(P)$^+$ and NAD(P)H and their synthetic derivatives. Because of the high analysis speed, HPCE can also be used to monitor continuously the course of coenzyme chemical modifications.

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