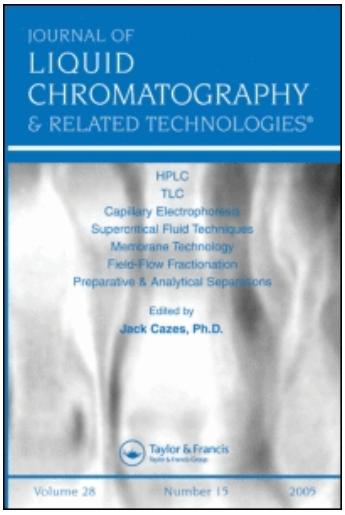

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Reverse Phase Liquid Chromatographic Analysis of the Impurities in Nicotinamide Adenine Dinucleotides

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REVERSE PHASE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE IMPURITIES IN NICOTINAMIDE ADENINE DINUCLEOTIDES

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ABSTRACT

Commercial preparations of several different nicotine adenine dinucleotides were examined by liquid chromatography on an octadecylsilane column (Margolis, S., et al., Clin. Chem., 22, 1322, 1976). Seven impurity peaks were detected in $NADP^+$, eight in NADPH, and five in NAD^+. The estimated purity of $NADP^+$ from different commercial suppliers varied from 89 to 95 percent. For NADPH the purity ranged from 77.5 to 96 percent and for NAD^+ from 90 to 93.5 percent. Preparations of NAD^+ contained AMP, ADPR, nicotinamide, and two unidentified impurities. The impurities found in $NADP^+$ and NADPH preparations did not correspond to compounds that we could identify. Four of the impurity peaks found in NADPH form under acidic storage conditions. Five of the impurity peaks observed in $NADP^+$ and three of the impurity peaks in NAD^+ form as products of alkali-catalyzed rearrangements.

INTRODUCTION

Nicotinamide adenine dinucleotides are known to be relatively unstable (2,3), particularly at neutral pH, and to give rise thereby to substances that inhibit oxido-reductase activity (4-9). The inhibitory substances may be generated in solutions of these dinucleotides (10-12). Several impurities are formed by

acid-catalyzed rearrangement of dinucleotides in their reduced form (13,14,17), or by base-catalyzed rearrangement of the oxidized forms (18,19). Other compounds form by the addition of ketones, aldehydes or α-keto acids to the nicotinamide moiety of oxidized dinucleotides (20). We have developed a reversed phase liquid chromatography (LC) method that provides a rapid separation and quantitation of such impurities from NADH (11,21). The LC method is used here for determining the purity of NAD^+, $NADP^+$, and NADPH, and to provide some elucidation of the source of these impurities.

METHODS AND MATERIALS

Samples of NAD^+, $NADP^+$, and NADPH were purchased from Boehringer Mannheim Biochemicals, Calbiochem, Sigma Chemical Co., and Pabst Laboratories. The dry solids were stored under vacuum in a dessicator at 4 °C. The samples were analyzed by the method of reverse phase LC that was previously reported (21). The chromatographic peaks were integrated with an electronic digital integrator.

RESULTS

Figures 1, 2, and 3 illustrate the number and chromatographic distribution of impurity peaks that can be detected in commercial NAD^+, $NADP^+$, and NADPH respectively by the LC method (21). Detection at 254 nm reveals all adenine nucleotides and dinucleotides; detection at 279 nm reveals the products that arise by acid-catalyzed rearrangement of NADH and NADPH (15,17), and detection at 340 nm and 350 nm reveals NADH, NADPH, and the products that arise by base-catalyzed rearrangement of NAD^+ and $NADP^+$. The proportion of each impurity was calculated from the integrated area of the peak recorded at 254 nm with the assumption that the extinction coefficients of dinucleotide impurities are similar at 254 nm to those for AMP, ADPR, and NADH (21).

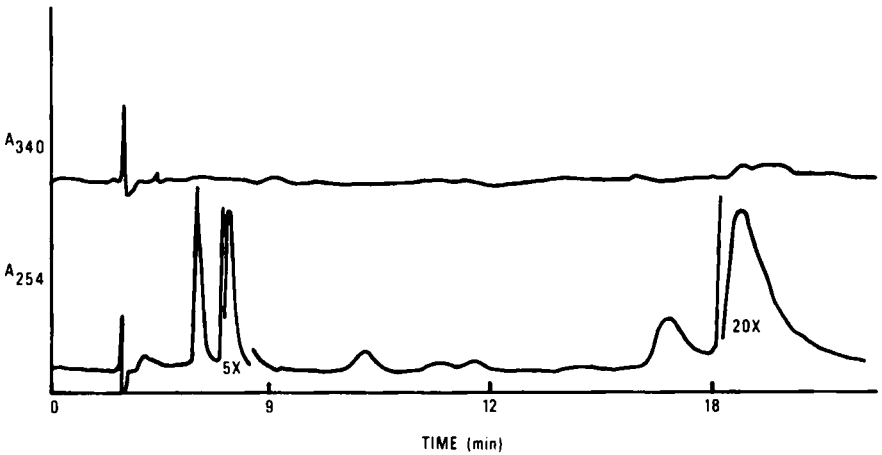

FIGURE 1. Chromatographic analysis of NAD^+ on an octyldecylsilane column. The concentration of NAD^+ (source 2) was about 0.6 mol/L. The solvent was 0.03 mol/L potassium phosphate (pH 7.05). The absorbance scale was 0.02A for 254 nm and 0.06A for 340 nm, and was attenuated as indicated by the values at the breaks in the chromatogram.

Purity of NAD^+

Table 1 summarizes an evaluation of NAD^+ preparations that were analyzed as received. These samples were purchased from four commercial suppliers. The content of NAD^+ ranged from 90 to 94 percent, based on its proportion of the total absorbance at 254 nm. The impurity peak with a retention time of 370 s is chromatographically identical to AMP, the peak at 440 s corresponds to ADPR, and the impurity at 1400 s behaves like nicotinamide (Tables 1 and 2). The peak at 780 s may correspond to the impurity in ADP ribose that is observed with the same retention time. The impurities at 780 s and 970 s do not absorb at 290 or at 340 nm, and their retention times do not correspond with other reference compounds tested (Table 2). However, the 780 s peak has the same relative

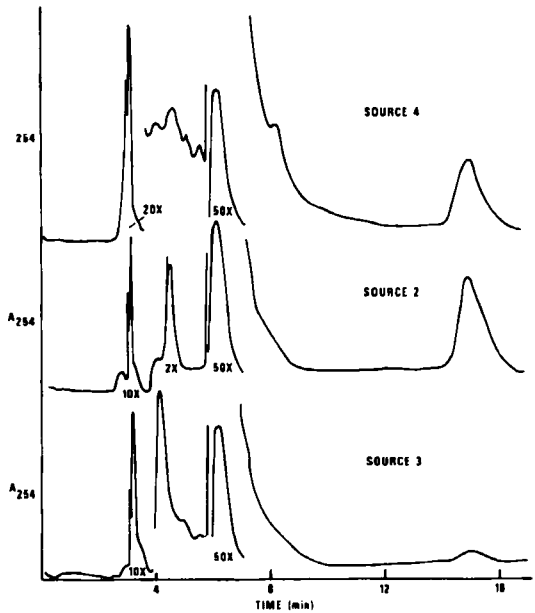

FIGURE 2. Chromatographic analysis of $NADP^+$ from different commercial sources.
The concentration of $NADP^+$ was about 0.6 mol/L. The solvent was 0.03 mol/L potassium phosphate (pH 7.05). The absorbance scale was 0.02A and was attenuated as indicated by the values at the breaks in the chromatograms.

retention time as an unidentified component that formed when NAD^+ was exposed to mild alkaline conditions. The origin of the peak at 970 s is unknown.

Purity of $NADP^+$

Table 3 summarizes our results on preparations of $NADP^+$ from three commercial suppliers. The content of $NADP^+$ ranged from 89 to 95 percent, based on proportions of the total absorbance at 254 nm. Seven impurities were detected. The peak with a retention time of 330 s corresponds to ADP. In this LC system, AMP and ADP ribose are not well separated from the $NADP^+$ and, if only

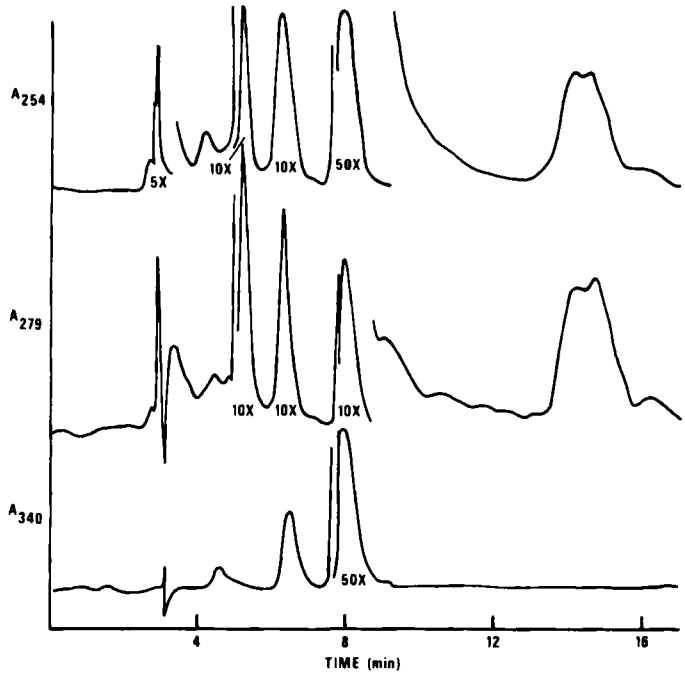

FIGURE 3. Chromatographic analysis of NADPH on an octadecylsilane column. The concentration of NADPH (source 3) was about 0.6 mol/L. The solvent was 0.03 mol/L potassium phosphate (pH 7.05). The absorbance scale was 0.02A for 250 nm, and 0.01A for 279 and 340 nm, and was attenuated as indicated by the values at the breaks in the chromatograms.

a small proportion of ADPR were present, it would not be detected. The other impurities detected at 254 nm, including the major impurity at 190 s, do not absorb at 290 or 340 nm, and their retention times do not correspond to any of our reference compounds. Thus these peaks do not correspond to the products of acid-catalyzed rearrangement of NADPH or of strong alkali-catalyzed rearrangement of $NADP^+$. However, treatment of $NADP^+$ with 0.1 mol/L NaOH for 30 minutes produced a series of peaks with the same retention times as the impurities.

TABLE 1

Composition of NAD^+ Preparations in Percentages of Total Chromatographic Peak Area at 254 nm

Peak Retention Time(s)	Source					
	1A	2	3	4	1B	1B[a]
370	1.9	1.0	1.1	1.4	0	0
440	4.0	5.2	5.3	4.3	6.6	52.1
780	0.6	<0.1	0.1	0.3	1.8	3.6
970	1.1	<0.1	1.0	0.3	1.6	1.6
1400	<0.1	0.4	<0.1	<0.1	0	6.8
1730	92.4	93.1	92.5	93.6	90.0	35.2

[a]after 30 minutes in 0.01 mol/L of NaOH.

TABLE 2

Elution of Nucleotides from an Octadecylsilane Column

	Retention Time (s)	Relative Retention Time (NADH=1)
NMN impurity	190	0.09
NMN	230	0.11
ADP impurity	290	0.14
ADP	320	0.16
$NADP^+$	370	0.18
AMP	370	0.18
ADPR	440	0.21
NADPH	490	0.24
AMP impurity	600	0.29
ADPR impurity	780	0.37
NICOTINAMIDE	1410	0.67
NAD^+	1730	0.83
NADH	2090	1.00
(CTHN)AD	4400	2.10

TABLE 3

Composition of $NADP^+$ Preparations in Percentages of Total Chromatographic Peak Area at 254 nm

Peak Retention Time(s)	Source			
	2	3	4	2[a]
190	2.7	5.9	9.4	39.8
250	<0.1	1.0	0	0.6
180	1.3	1.0	0.2	2.4
300	<0.1	<0.1	<0.1	<0.1
330	0	<0.1	0.1	1.0
370	94.8	92.8	89.1	50.7
490	0	0	0.2	0.6
890	1.0	0.1	0.8	4.9

[a] 0.1 mol/L NaOH for 30 minutes.

Purity of NADPH

Table 4 provides a summary of the LC results on preparations of NADPH from three suppliers. The content of NADPH ranged from 77 to 96 percent, by relative absorbance at 254 nm. Eight peaks were detected. None corresponds to any of our reference compounds. The ratio $A_{279\ nm}/A_{254\ nm}$, found for three of the peaks, was five times larger than for NADPH (figure 3), which is a characteristic of products of acid-catalyzed rearrangement (15, 17). Exposure of NADPH to 0.1 mol/L phosphate buffer (pH 7.0) led to the formation of peaks having retention times and spectral properties like the impurities in the commercial samples (Table 5). The peak at 190 s formed simultaneously with the peak at 890 s. The origin of the peaks at 260 s and 300 s is obscure. At 340 nm, two peaks in addition to NADPH were observed, and neither appeared to chromatograph exactly with the peaks observed at 254 nm. Finally, the instability of NADPH at pH 7.05 is

TABLE 4

Composition of NADPH Preparations in Percentages of Total Chromatographic Peak Area at 254 nm

Peak Retention Time(s)	Source		
	2	3	4
190	0.9	0.7	1.7
200	<0.1	0.5	0
260	0.2	0.4	0.1
300	<0.1	<0.1	0
320	0.7	0.3	0.5
380	2.1	0.8	8.9
490	95.9	95.7	77.6
560	<0.1	1.4	0
890	0	0	1.5

TABLE 5

The Formation of Products of Acid-Catalyzed Rearrangement of NADPH[a] in Percentages of Total Peak Area at 254 nm

Time (min)	Temp (°C)	Peak Retention Time(s) 190	260	300	320	380	490	890
0	22	0.5	1.4	0.8	1.2	6.2	89.7	0
60	22	0.8	1.3	0.8	4.3	9.8	82.4	0.5
90	22	0.9	1.4	0.8	5.8	11.1	79.9	0.8
120	22	1.1	1.4	0.7	7.0	12.0	76.7	1.1
180	22	1.3	1.3	0.7	9.3	14.2	71.5	1.6
0	4	0.6	1.5	0.9	1.2	6.0	89.4	0
30	4	0.7	1.4	0.9	1.5	6.8	88.7	0
60	4	0.7	1.4	1.0	1.7	7.4	87.8	0.03
90	4	0.9	1.4	0.9	1.9	7.7	87.2	0.07
120	4	0.9	1.4	0.9	2.4	8.4	85.6	0.1
180	4	0.7	1.3	0.8	2.9	8.8	85.0	0.2

[a] [NADPH] = 2 mg/mL 0.1 mol/L phosphate buffer pH 7.05; sample size 10 µL.

striking. At 22 °C, 18 percent of the NADPH degraded within three hours. At 4 °C, 4.4 percent of the NADPH was destroyed in three hours (Table 5).

Purity of the Reference Compounds

The compounds used for reference exhibited significant proportions of impurities. Both the ADP and ADPR contained significant proportions of AMP as well as other unidentified substances. Only nicotinamide appeared to be relatively pure.

DISCUSSION

The occurrence of impurities which modify the interaction of nicotinamide adenine dinucleotides with proteins makes it necessary to evaluate the purity of the dinucleotides before they are used in binding studies, in assessing the accuracy of kinetic measurements, or in deciding whether an inhibitor assay truly reflects the absence of all inhibitors or activators.

Our method of reverse phase LC is very efficient for the qualitative and quantitative evaluation of the purity of nicotinamide adenine dinucleotides. Less than one hour is required to complete the separation. Only 10 μg of sample is required. The presence of AMP, ADPR, NAD^+, acid-catalyzed rearrangement products, and other substances that are enzyme inhibitors has been demonstrated, and their relative amounts in NADH were previously estimated by this method (11,21). The presence of enzyme inhibitors in NAD^+ (5,6) and their formation under alkaline conditions (19), and the presence of enzyme inhibitors in NADPH (4) have been reported.

A survey by reverse phase LC of the purity of commercial samples of NAD^+, $NADP^+$, and NADPH has shown that the preparations sampled were not pure. Based on the proportion of total absorbance at 254 nm, samples were less than 96 percent pure. These estimations of purity are not based on weight percent, because we do not know the identities, molecular weights, or extinction

coefficients of the impurities. One sample of NADPH (source 4) was 23 percent impure. For most samples from 5 to 10 percent of the total A_{254} is due to impurities. NAD^+ contained AMP, ADPR, nicotinamide, an impurity that also forms during mild alkali-catalyzed rearrangement, and an impurity of unknown origin. None of these impurities is a product of acid-rearrangement of NADH because each lacked the characteristic absorbance at 279 nm and 290 nm (15,22); and none was the product of rearrangement of NAD^+ in strong alkali (18,19), or the adduct of keto acids, aldehydes, or ketones with NAD^+ (20) since none had the characteristic absorbance at 340-350 nm (18,20). Some of these impurities were absent in at least one sample of each kind of dinucleotide we examined; hence the impurities are not artifacts of chromatography.

$NADP^+$ and NADPH can undergo chemical modifications similar to NAD^+ and NADH (2,4,20). The presence of ADPR in $NADP^+$ or in NADPH is not likely since the type of chemical reaction which forms ADPR from NAD^+ and NADH would produce ADPR-2'-P from $NADP^+$ and NADPH (4). The additional phosphate group in ADPR-2'-P should decrease the product's retention time relative to ADPR just as the second phosphate in ADP decreases the retention time of ADP relative to AMP (Table 2). The small proportionate absorbance at 279 nm and 340 nm of the 190 s peak indicates that this peak may be an adenine nucleotide, which is more polar than ADP. Its formation during acid rearrangement of NADPH (Table 5) strongly indicates that it is ADPR-2'-P. Williams (4) demonstrated that ADPR-2'-P is a competitive inhibitor of dihydrofolate reductase and suggested that acid-catalyzed rearrangement products of NADPH may be present and might also affect the enzymatic activity. It is evident from our comparative studies that significant proportions of acid-catalyzed rearrangement products, including ADPR-2'-P, are present in commercial NADPH. Spectral analysis indicates that the two products at 320 s and 380 s have spectral properties which, by analogous studies on NADH (15-17), may be predicted to be the NADPH analogs of

β-6-hydroxytetrahydronicotinamide adenine dinucleotide [(6HTN)AD]. Their retention times relative to NADPH and their formation at pH 7.05 follow a pattern similar to that observed for the formation of (6HTN)AD from NADH (15).

The majority of the impurities in $NADP^+$ appear to be generated by a mild-base catalyzed rearrangement (Table 3); whereas the majority of the impurities in NADPH appear to be generated by an acid-catalyzed rearrangement (Table 5). Studies on the stability of NADH (unpublished) indicate that products of acid-catalyzed rearrangement may form during lyophilization of pure NADH in ammonium bicarbonate buffer below pH 10. It is highly likely that the corresponding impurities in $NADP^+$ and NADPH are produced during similar processing of these materials.

The instability of NADPH at pH 7 between the temperatures of 4 and 22 °C is much greater than that of NADH (2,15). Activity measurements and binding studies are often performed at or near this pH and in this temperature range. It is evident from our studies that on storage under these conditions for several hours NADPH is chemically modified. Furthermore, products such as ADPR-2'-P have been shown to inhibit dihydrofolate reductase (4) and may also inhibit other enzymes. The inhibitory properties of the other products remain unknown, however their probable formation during prolonged binding studies (23,24) and their possible competition for binding sites could be significant.

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(1) Identification of any commercial product does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material or equipment identified is necessarily the best available for the purpose.

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