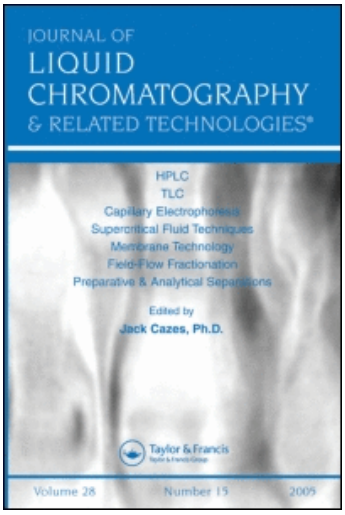

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Amperometric Methods for Oxidoreductase Enzymes Based on Liquid Chromatography with Electrochemical Detection. Alcohol Dehydrogenase

Gregory C. Davis[a]; Karey L. Holland[a]; Peter T. Kissinger[a]
[a] Chemistry Department, Purdue University, West Lafayette, Indiana

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AMPEROMETRIC METHODS FOR OXIDOREDUCTASE ENZYMES BASED ON LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION. ALCOHOL DEHYDROGENASE.

Gregory C. Davis, Karey L. Holland, and Peter T. Kissinger
Chemistry Department
Purdue University
West Lafayette, Indiana 47907

ABSTRACT

Nicotinamide adenine dinucleotide (NAD) is an important cofactor in a number of oxidoreductase enzyme systems. The detection and quantitation of its reduced form (NADH) is the basis for a number of methods which determine both substrates and enzyme activity. Although electrochemical techniques have shown promise in this area, they have suffered from a lack of selectivity. Liquid chromatography with electrochemical detection (LCEC) provides the selectivity and the sensitivity required. The use of alcohol dehydrogenase (ADH) for blood alcohol detection is investigated as a model system.

INTRODUCTION

A large number of enzymatic methods have been developed which employ the use of the cofactor β-nicotinamide adenine dinucleotide (NAD) and its reduced form, NADH (1-3). The production of NADH has been used to determine both the concentration of substrates and the activity of enzymes. NADH is primarily quantitated by spectrophotometry or fluorescence. Recently, Christian, Blaedel, Elving, Olson and others have clearly demonstrated the potential of electrochemistry in the detection of enzymatically generated NADH (4,5).

NADH is electrochemically oxidized in a $2e^-$ reaction (Figure 1) to the parent nucleotide NAD (6,7). Consequently it is possible to indirectly quantitate the amount of substrate in a sample by monitoring the NADH oxidation. Amperometric procedures based on this idea have suffered from a lack of selectivity. The NADH oxidation current is often superimposed on a significant background caused by more easily oxidized biological components (8,9). Electroactive contaminants, such as uric acid or ascorbic acid, which are contributed from serum, make it difficult, if not impossible, to accurately detect small changes in NADH concentration using simple amperometry.

The general LCEC approach described here offers a marked improvement over previous amperometric procedures. By combining the powerful separating ability of the microparticulate C-18 stationary phase with the selectivity of the electrochemical detector, the problem of detecting small amounts of NADH in the presence of high concentrations of other electroactive contaminants is eliminated. To illustrate the potential of this approach, the enzyme alcohol dehydrogenase (EC 1.1.1.1) was chosen as a model system. A blood-ethanol method is described which utilizes the following reaction,

$$CH_3CH_2OH + NAD^+ \xrightarrow[pH\ 8.8]{ADH} CH_3CHO + NADH + H^+ .$$

MATERIALS AND METHODS

Chemicals

Dihydronicotinamide adenine dinucleotide (NADH, Grade III), nicotinamide adenine dinucleotide (NAD^+, Grade III), alcohol dehydrogenase (ADH, from yeast) and Tris (hydroxymethyl) amino methane (Trizma Base and Trizma HCl) were obtained from Sigma Chemical Co. (St. Louis, MO). Rossville Gold 200 proof ethanol was purchased from IMC Chemical Group, Inc. (Terre Haute, IN). Semicarbazide HCl was acquired from Eastman Kodak (Rochester, NY).

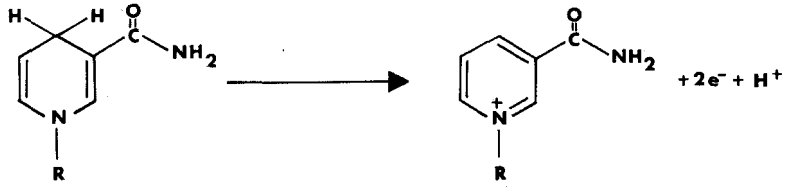

Figure 1. The electrochemical oxidation of NADH.

All other chemicals were reagent grade quality. Doubly distilled water was used in preparing all solutions.

Reagents

Pyrophosphate buffer, 75 mM, pH 8.8 is prepared by weighing 33.4 g of $Na_4P_2O_7 \cdot 10\ H_2O$, 8.4 g of semicarbazide HCl and 1.6 g of glycine into a large beaker and dissolving these solids in 800 mL of water. The pH is adjusted to 8.8 by slow addition of 3 M NaOH. When the proper pH is attained, the solution is diluted to 1 liter with water. Pyrophosphate buffer is used because it complexes heavy metal ions which are known to inactivate the yeast enzyme (14). When not in use,this solution should be stored at 5°C to inhibit the growth of microorganisms.

A 3 mM NAD^+ solution is prepared by dissolving 108.53 mg of NAD^+ in fifty mL of the pyrophosphate buffer solution. This reagent remains stable for 1 week if stored in a refrigerator.

The alcohol dehydrogenase obtained from Sigma Chemical Co. had a stated activity of 312 U/mg solid (10). A solution with an activity of 3.1 U/μL can be prepared by adding 50 mg of the solid in 5 mL of water. This solution is stable for approximately 6 months if kept frozen (10). The 5 mL are divided between 3 small vials so as to avoid numerous deleterious freeze-thaw cycles on the same solution.

A diluent buffer is required since the amount of NADH generated in the reaction is so large that it is necessary to dilute the sample before injecting onto the LC. This ensures that

the amount of NADH is in the linear range of the detector. A 0.1 M pH 8.5 Tris buffer serves this purpose well. It is prepared by dissolving 0.44 g of Trizma HCl and 0.872 g of Trizma base in 100 mL of water.

A 0.01 M Hg^{+2} solution is used in the final step of the procedure to ensure that the enzyme reaction is stopped (see Results and Discussion section). A 1.0 M solution of $HgCl_2$ is initially prepared by dissolving 27.15 g of $HgCl_2$ in 100 mL of H_2O. This stock solution is diluted one hundredfold to get the 0.01 M Hg^{+2}.

To check the linearity of the detector, NADH standards are prepared. A stock solution is prepared by weighing out 29.15 mg of the disodium salt (25 mg of the free NADH) into a 50 mL volumetric flask and dissolving the solid in a 0.1 M pH 8.0 phosphate buffer. A range of concentrations is made by diluting this stock solution (100 ng/μL) with the appropriate amount of buffer. The standards range from 15 ng/μL to 0.5 ng/μL. Since a 20 μL injection loop was used, this corresponds to 300 ng to 10 ng injected. Figure 2 illustrates the linearity of the detector. These solutions are stable for at least 3 days.

Ethanol standards are prepared which represent the range of ethanol found in blood after a 1 to 10 dilution (11). A stock solution is prepared by pipetting 10 mL of absolute ethanol into 1 liter of water. The resulting solution has a concentration of 7.89 g/L. By the appropriate dilutions, the ethanol standards range from 0.473 g/L to 0.0395 g/L. These standards are stable for several weeks if kept frozen.

Instrumentation

The instrumentation consisted of a model LC-50 liquid chromatograph with a TL-5 glassy carbon electrode purchased from Bioanalytical Systems Inc. (W. Lafayette, IN). The potential of the working electrode was maintained at +0.750 V vs. a Ag/AgCl

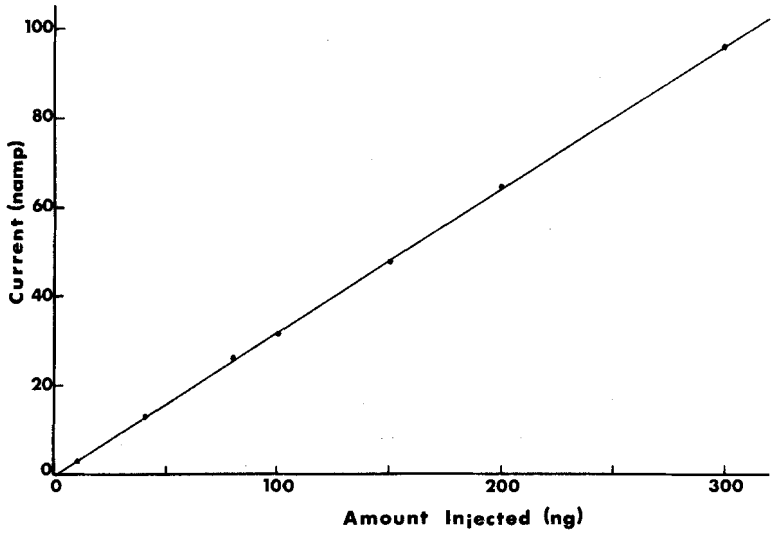

Figure 2. Peak current response as a function of the amount injected.

reference electrode. The separations were made on a 15 cm x 4 mm i.d. stainless steel column packed with 10 µm RP-18 stationary phase obtained from E.M. Laboratories (Darmstadt, Germany). The mobile phase was a pH 5.25 0.15 M phosphate/citrate buffer containing 4.5% methanol. The flow rate was 1 mL/min in all instances.

Procedure

Add 3 mL of the 3 mM NAD^+ solution to a 10 mL test tube. Pipet 30 µL of the ADH to this and swirl the mixture briefly with a Vortex mixer (Fischer Scientific). Finally, add 100 µL of the ethanol standard or diluted serum sample to the mixture and briefly swirl it to ensure complete mixing. Incubate this solution for 20 minutes to allow the reaction to go to completion. All incubations are carried out in a water bath at 25°C. When the incubation period is over, remove a 100 µL aliquot and place it in a 3 mL test tube which contains 2 mL of the Tris buffer and 100 µL of the 0.01 M Hg^{+2} solution. This last mixture is briefly swirled on a Vortex mixer prior to injection onto the LC column.

A reagent blank is always examined with each set of samples. The NADH present in the reagent blank is subtracted from all of the samples to correct for any trace amounts of ethanol that might be present in the reagents, e.g. alcohol dehydrogenase has a trace amount of ethanol in the lyophilized enzyme (10).

RESULTS AND DISCUSSION

Initial experiments used ethanol standards in order to evaluate the method under ideal matrix-free conditions. The results are illustrated in Figure 3. The linearity is demonstrated over a range of ethanol concentrations, which, as previously mentioned, were chosen to represent serum alcohol levels after dilution. Further evidence of the method's accuracy is shown in Table 1.

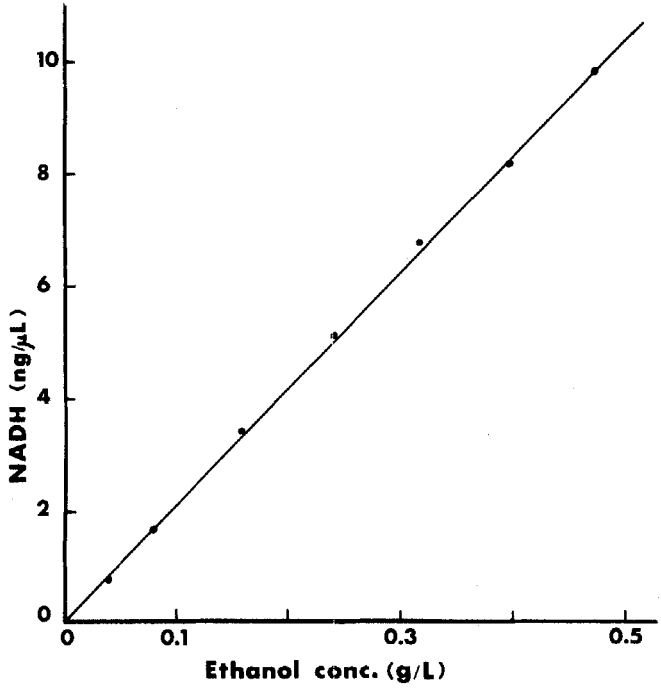

Figure 3. Enzymatic generation of NADH from various ethanol standards.

TABLE 1

Ethanol Std. (g/L)	Theoretical (ng/µl)	Experimental* (ng/µl)
0.0395	0.823	0.784
0.0789	1.65	1.64
0.158	3.29	3.41
0.237	4.94	5.14
0.316	6.58	6.81
0.395	8.23	8.22
0.473	9.88	9.83

Slope of theoretical values: 20.86 (correlation coefficient 1.00)
Slope of experimental values: 20.88 (correlation coefficient 1.00)
*All values background corrected

The table lists the ethanol standards, the theoretical concentration of NADH which should be produced, and the actual NADH concentration found in the sample. A linear regression analysis of the data indicates that the slope of the lines for the theoretical and experimental values are statistically indistinguishable.

When the procedure is applied to human serum samples spiked with ethanol, similar results are obtained. Table 2 compares the theoretical values to those found experimentally in the serum samples.

As with the previous results, a linear regression analysis of the two sets of data demonstrates that the slopes for the two lines are statistically indistinguishable.

A typical chromatogram of NADH produced from the enzymatic oxidation of ethanol is shown in Figure 4. The large void volume response is due in part to oxidizable serum contaminants, but is primarily accounted for by excess semicarbazide. The semicarbazide is added to the pyrophosphate buffer to complex the acetaldehyde and pull the reaction firmly to the right. The separation of NADH from the void volume response is more than adequate and the retention time of the NADH peak matches that of a standard.

TABLE 2

Ethanol Std. (g/L)	Theoretical*1 (ng/µl)	Experimental*2 (ng/µl)
0.0395	0.798	0.791
0.0789	1.59	1.79
0.158	3.19	3.35
0.237	4.78	5.02
0.316	6.38	6.37
0.395	7.78	8.22
0.473	9.55	9.70

Slope of theoretical values: 20.20 (correlation coefficient 1.00)

Slope of experimental values: 20.35 (correlation coefficient 1.00)

*1The difference between the theoretical values in tables 1 and 2 are due to a different dilution factor.

*2All values background corrected

When using LC for trace organic determinations positive identification of an eluted compound can be difficult. In addition, since most LCEC determinations involve nanogram amounts of injected materials, it is virtually impossible to obtain spectral information from collected fractions.

One means of further confirming the presence of a compound is by plotting hydrodynamic voltammograms for specific chromatographic zones. The hydrodynamic voltammograms are constructed by making repeated injections of a sample at different potentials and plotting the peak height vs. the applied potential. In order to facilitate comparison of the voltammogram with that of a standard, it is useful to ratio all the peak heights to that measured at the most positive potential. The resulting normalized current function, ϕ, is plotted vs. the applied potential. If the suspected compound in a particular chromatographic zone and a standard are truly the same, then the curves will be identical. Figure 5 illustrates this point dramatically. Three hydrodynamic voltammograms are generated: A for an NADH standard, B for NADH generated using an ethanol standard, and C for NADH generated

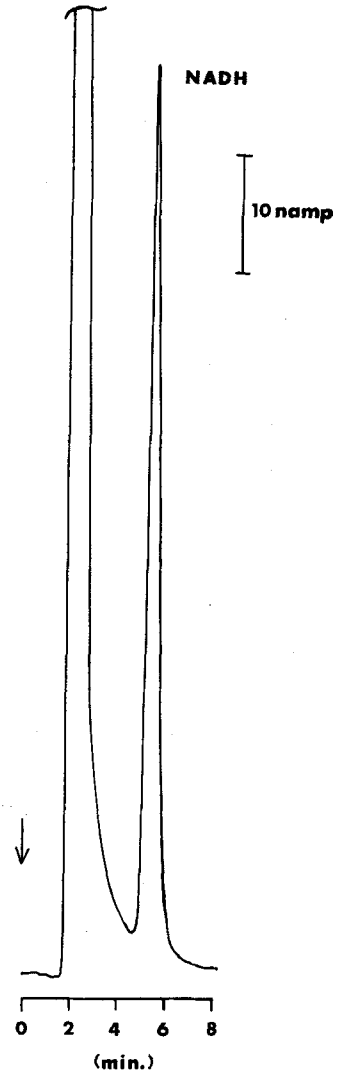

Figure 4. This chromatogram illustrates the applicability of the method. A 100 µl aliquot of a coroner blood sample (diluted 1:10) which was taken from a driver involved in a one car accident, was tested using the LCEC procedure.

from a serum sample containing ethanol. It is obvious that all three curves are identical and that NADH is the compound measured.

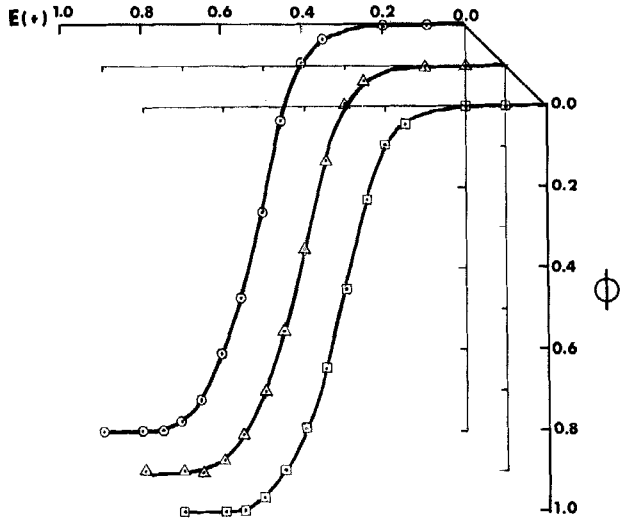

Figure 5. Hydrodynamic voltammograms for NADH from three sources. (See text for details).

The pH of the mobile phase used here is 5.25. NADH is acid labile and can decompose into a number of products (12,13). Of the reverse phase LC methods that have been published, many have used a mobile phase pH of 7 or greater. A pH of 7 is usually regarded as near the upper limit for reverse phase systems. The poor chromatographic results (broad, tailing peaks) that were obtained in the published reports indicates that the columns may have been adversely affected by the high pH. The pH of our mobile phase is a compromise between the decomposition of NADH and the stability of the reverse phase material. A decomposition study of NADH at pH 5.25 reveals that for the short period of time that NADH spends in the column, a negligible amount decomposes. As a result, the choice of this mobile phase is justified.

As mentioned briefly in the procedure, heavy metal ions will dissociate ADH into inactive subunits (14). Normally buffers such as pyrophosphate are used to control the incubation pH as it can complex these ions and prevent the dissociation of the enzyme. However, we found it necessary to make use of these

dissociative effects by adding Hg^{+2} ions to the diluent buffer, and thereby deliberately dissociating the small amount of enzyme present in this final solution. Tris is used as the diluent and does not complex Hg^{+2} ions. The addition of Hg^{+2} to the diluent was incorporated into the procedure after it was observed that on repeated injection of a given sample, the NADH response would increase with time. This phenomenon was observed in several buffer systems that were used to dilute the incubation mixture (carbonate, phosphate, pyrophosphate, Tris, and McIlvaine were all tested). When the Hg^{+2} ions were added to the diluent, the NADH response did not increase. It might be thought that all of the ethanol had not reacted, but the data in Tables 1 and 2 indicates that it has. Additional evidence in this regard lies in the observation that when an aliquot is taken immediately after the incubation is finished (20 minutes) and again an hour later, the initial response for each sample is identical. Yet without the addition of Hg^{+2}, on reinjection the NADH response would increase. The possibility that trace contiminants which could serve as ADH substrates and reduce the remaining NAD (which is present in excess) is currently being investigated.

A reproducibility study was conducted using ten replicate analyses of a 0.316 g/L ethanol standard. The precision of the method in terms of relative standard deviation was 2.5%.

The alcohol dehydrogenase system serves as an effective model in demonstrating the potential of liquid chromatography for the analysis of enzymatically generated NADH. With the use of the sensitive amperometric detector, a general assay scheme can be developed (Figure 6). This approach can be applied to the analysis of substrates or enzymes, as long as the cofactor can be separated from other oxidizable components. Currently methods are being developed which are based on this approach for serum lactate and lactate dehydrogenase.

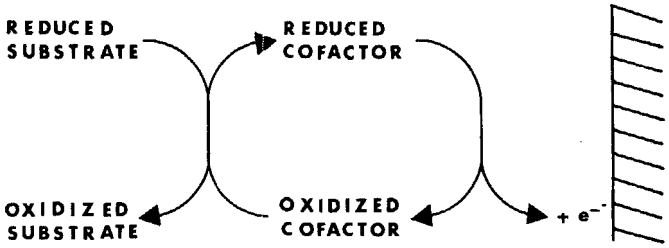

Figure 6. General detection scheme for enzymatic systems utilizing an electroactive cofactor.

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REFERENCE LIST

1. Kuan, J. W., Kuan, S. S., and Guilbault, G. G., The Immobilized-Enzyme Stirrer, Anal. Chim. Acta, 100, 229 (1978).

2. Stambaugh, R. and Post, D., A Spectrophotometric Method for the Assay of Lactic Dehydrogenase Subunits, Anal. Biochem., 15, 470 (1966).

3. Brooks, L. and Olken, H. G., An Automated Fluorometric Method for Determination of Lactate Dehydrogenase in Serum, Clin. Chem., 11, 748 (1965).

4. Heineman, W. R. and Kissinger, P. T., Analytical Electrochemistry: Methodology and Applications of Dynamic Techniques, Anal. Chem., 50, 166R (1978).

5. Kissinger, P. T., Analytical Electrochemistry: Methodology and Applications of Dynamic Techniques, Anal. Chem., 48, 17R (1976).

6. Aizawa, M., Coughlin, R. W., and Charles, M., Electrochemical Regeneration of Nicotinamide Adenine Dinucleotide, Biochim. et Biophys. Acta, 385, 362 (1975).

7. Braun, R. D., Santhanam, K. S. V., and Elving, P. J., Electrochemical Oxidation in Aqueous and Nonaqueous Media of Dihydropyridine Nucleotides NMNH, NADH, and NADPH, J. Am. Chem. Soc., 97, 2591 (1975).

8. Thomas, L. C. and Christian, G. D., Voltammetric Measurement of Reduced Nicotinamide-Adenine Nucleotides and Application to Amperometric Measurement of Enzyme Reactions, Anal. Chim. Acta, 78, 271 (1975).

9. Wallace, T. C., Leh, M. B., and Coughlin, R. W., Amperometric Assay of Coenzyme-Dependent Oxidoreductase Enzymes in a Flow-Through Cell, Biotech. and Bioeng., 19, 901 (1977).

10. Sigma Chem. Co. Bulletin - Product No. A-7011.

11. Cheng, F. S. and Christian, G. D., Enzymatic Determination of Blood Ethanol, with Amperometric Measurement of Rate of Oxygen Depletion, Clin. Chem., 24, 621 (1978).

12. Margolis, S. A., Yap, W. T., Mathews, B. and Schaffer, R., Reverse-Phase Liquid Chromatographic Analysis of the Acid-Catalyzed Rearrangement of NADH, Separation of New Products and an Analysis of the First Two Reactions of the Rearrangement, J. Liq. Chromatogr., 1, 669 (1978).

13. Miksic, J. R. and Brown, P. R., Reactions of Reduced Nicotinamide Adenine Dinucleotide in Acid: Studies by Reversed-Phase High-Pressure Liquid Chromatography, Biochemistry, 17, 2234 (1978).

14. Townshend, A. and Vaughan, A., Applications of Enzyme-Catalyzed Reactions in Trace Analysis-VI. Determination of Mercury and Silver by Their Inhibition of Yeast Alcohol Dehydrogenase, Talanta, 17, 299 (1970).