Electrophoretically mediated microanalysis of ethanol

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ABSTRACT

Capillary electrophoresis was used to determine ethanol by the methodology of electrophoretically mediated microanalysis (EMMA). In EMMA, spatially distinct analyte and analytical reagent zones of differing electrophoretic mobility are merged under the influence of an electric field, and the resulting product is transported to the detector. The enzymatic oxidation of ethanol to acetaldehyde by alcohol dehydrogenase was utilized, and the concurrent reduction of NAD^+ to NADH was monitored at 340 nm as a measure of the quantity of ethanol injected. Quantitation using an internal standard and normalization for peak migration time yielded a R.S.D. of 2.7%, and the linear range extended to that quantity of ethanol which could be reacted prior to passing by the detection window. Comparison of the EMMA technique to the Sigma spectrophotometric procedure revealed that the two methods do not yield significantly different values for the determination of ethanol. The EMMA method offered the advantages of electrophoretic mixing and miniaturization.

INTRODUCTION

Capillary electrophoresis has traditionally exploited the variability in electrophoretic mobility among charged species as a method to separate such substances. However, diversity in migration velocity under the influence of an applied electric field also offers the capability of electrophoretically mixing spatially distinct zones of chemical reagents [1–4]. As we have recently described [4], capillary electrophoretic systems offer potential for performing ultramicroassays using a methodology known as electrophoretically mediated microanalysis (EMMA). In EMMA, electrophoretic mixing is utilized to merge zones containing the analyte and analytical reagents; the reaction is then allowed to proceed either in the presence or absence of the applied electric field; and, finally, the detectable product is transported under the influence of an applied potential to the detector.

This paper demonstrates the use of EMMA as an analytical technique for the determination of substrates by enzymatic reactions. The enzymatic system chosen for this study is the catalytic oxidation of ethanol to acetaldehyde by alcohol dehydrogenase (ADH; EC 1.1.1.1):

$$CH_3CH_2OH + NAD^+ \xrightarrow{\text{ADH}} CH_3CHO$$

$$+ NADH + H^+$$
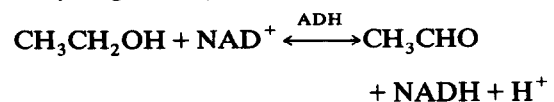
The concurrent reduction of the coenzyme NAD^+ to NADH can be monitored by the increase in absorbance at 340 nm as a measure of the extent of reaction and, therefore, the amount

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of ethanol contained in the sample. ADH-based enzymatic methods [5] are commonly used for the determination of alcohol in many clinical laboratories. The equilibrium, which lies far to the left at neutral pH, can be forced to the right by buffering at alkaline pH and by trapping the acetaldehyde with an agent, such as hydrazine or semicarbazide.

EXPERIMENTAL

Instrumentation

All EMMA assays were performed using a BioFocus 3000 capillary electrophoresis system from Bio-Rad Labs (Hercules, CA, USA). Poly-imide-coated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. × 180 μm O.D. were utilized. The capillaries were of 24 cm total length with a separation length (distance from injection inlet to detection window) of 19.4 cm.

The spectrophotometric determinations were performed using a Spectronic 20D spectrophotometer (Milton Roy, Niagara Falls, NY, USA) operated at 340 nm.

Chemicals

Yeast alcohol dehydrogenase (YADH; 380 units/mg solid as assayed by Sigma), nicotinamide adenine dinucleotide (NAD$^+$), glycine buffer solution (0.5 M, pH 9.0) containing hydrazine trapping agent, and p-nitrophenol were purchased from Sigma (St. Louis, MO, USA). Absolute ethanol and mesityl oxide (neutral marker used in determination of electrophoretic mobilities) were purchased from Midwest Solvents of Illinois (Pekin, IL, USA) and Aldrich (Milwaukee, WI, USA), respectively. Glycine running buffer (50 mM, pH 9) was prepared by diluting the 0.50 M glycine buffer solution with degassed, double-distilled, deionized water. The analytical reagent/running buffer solution was prepared by dissolving YADH (200 units/ml for Figs. 1-3 and 5; 50 to 400 units/ml for Fig. 4) and NAD$^+$ (10 mM) in the running buffer solution and adjusting to pH 9.0 with 1 M NaOH. Ethanol standards were prepared by diluting absolute ethanol with appropriate amounts of degassed, double-distilled, deionized

water and adding p-nitrophenol as an internal standard.

EMMA procedures

The capillaries were conditioned with 10-min rinsings of 1 M NaOH and running buffer prior to use. The capillary and the buffer reservoirs were filled by pressure with analytical reagent/ buffer solution, and a plug of sample was then hydrodynamically injected by the application of pressure for a pressure · time constant of 1 p.s.i. · s. The assay was effected by applying an electric field (125 V/cm for Figs. 1 and 2; 300 V/cm for Figs. 3-5) and monitoring the absorbance electropherogram at 340 nm. The capillary was thermostatted by circulating water at 25°C throughout the assay. Between determinations the capillary was purged for 30 s with 0.1 M KOH followed by 60 s with analytical reagent/ running buffer solution.

Spectrophotometric procedures

The spectrophotometric determination of ethanol was performed as described in the manual [6] contained with the Sigma Diagnostics Alcohol (Ethanol) determination kit.

RESULTS AND DISCUSSION

EMMA methodology

Conventional reaction-based chemical analysis requires four processes: (1) analyte and analytical reagent metering, (2) initiation of reaction, (3) control of reaction conditions and product formation and (4) detection of species whose production or depletion is indicative of the quantity of analyte of interest. Capillary electrophoretic systems, as employed in the EMMA determination of ethanol, are capable of executing each of these tasks [4]. In the EMMA determination of ethanol, the analytical reagents were metered by filling the capillary and the buffer reservoirs with pH 9 glycine buffer solution containing ADH and NAD$^+$, and the analyte was then metered by injecting a plug of ethanol solution at the anodic inlet.

Electrophoretic mixing of the analyte and analytical reagents was initiated by the application of an electric field. The reagent zones

TABLE I

ELECTROPHORETIC MOBILITIES OF CHEMICAL SPECIES AT pH 9

Chemical species	Electrophoretic mobility $(cm^2 V^{-1} s^{-1})$
Ethanol	0
YADH	$-1.6 \cdot 10^{-4}$
NAD$^+$	$-1.2 \cdot 10^{-4}$
NADH	$-2.3 \cdot 10^{-4}$
p-Nitrophenol (internal standard)	$-3.0 \cdot 10^{-4}$

migrated at differential rates under the influence of an electric field as dictated by their respective electrophoretic mobilities listed in Table I. Neutral ethanol migrated with the bulk electroosmotic flow toward the cathode while ADH and NAD$^+$ were each negatively charged at pH 9 and electrophoresed against the electroosmotic flow. Consequently, the ethanol zone interpenetrated the adjacent zones of ADH and NAD$^+$. Since the analytical reagents were maintained as a continuous stream within the capillary and the buffer reservoirs, upon electrophoretic mixing ethanol remained engaged with the ADH and NAD$^+$ zones throughout its traversal of the capillary.

The reaction phase of this assay was performed under the influence of a constant applied potential. As the ethanol zone incubated within the analytical reagent zones, NADH was produced as dictated by the kinetics of YADH [7,8]. Because NADH is negatively charged, it was continually transported from the vicinity of the reaction under the influence of the constant applied potential. However, since the magnitude of its electrophoretic mobility is less than that of the electroosmotic flow, NADH migrated toward the detection window. We have previously described the effects of kinetics and differential electrophoretic mobility upon the observed concentration profile of the NADH [4]. Typical electropherograms obtained for EMMA determinations of ethanol are depicted in Fig. 1.

Clinical substrate assays are frequently based upon end-point methods in which the reaction is allowed to essentially reach completion prior to
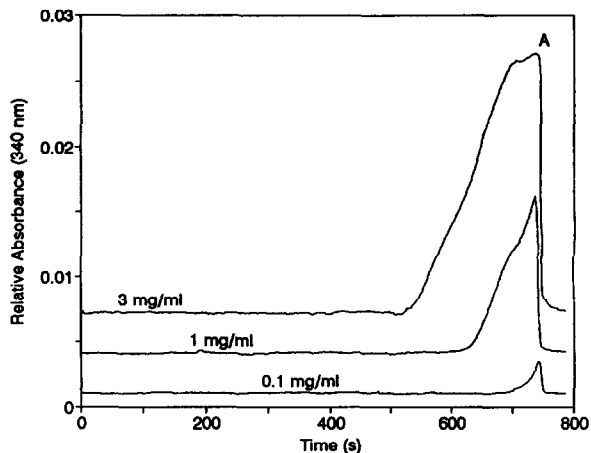

Fig. 1. EMMA determinations of 3, 1 and 0.1 mg/ml samples of ethanol; (A) NADH accumulation due to diffusional interpenetration at reagent interfaces prior to application of electric potential. For conditions see text.

taking a spectrophotometric reading. In the EMMA methodology, an end-point determination requires that all of the ethanol react prior to passing by the detection window. NADH formed after the ethanol zone passes by the detection window is not observed. This truncation effect places the upper limit on the linear range of the technique [4]. Assuming electrophoretic mixing is rapid, the total reaction time t_{rxn} available to fully deplete the substrate is equal to the time required for the ethanol to migrate from the injection point to the detection window:

$$t_{rxn} = \frac{l}{(\mu_{ep,EtOH} + \mu_{eo})E} \quad (1)$$

where l is the separation length of the capillary, $\mu_{ep,EtOH}$ is the electrophoretic mobility of ethanol, μ_{eo} is the electroosmotic flow and E is the electric field strength. Based upon the experimental electroosmotic flow of $4.4 \cdot 10^{-4} cm^2 V^{-1} s^{-1}$ and electric field strength of 125 V/cm, the assays depicted in Fig. 1 offered an available substrate incubation time of approximately 350 s.

Since NADH had a lower transport velocity than ethanol, the first NADH formed (due to diffusional interpretation at the interfaces of the adjacent reagent zones prior to the application of the electric field [1,4]; indicated by A in Fig. 1), was the last to be detected. The first NADH

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which could be detected was that which formed as any remaining unreacted ethanol passed by the detection window. Therefore, there was a detection time window t_{det} during which NADH could be observed:

$$\frac{l}{(\mu_{ep,EtOH} + \mu_{eo})E} \leqslant t_{det} \leqslant \frac{l}{(\mu_{ep,NADH} + \mu_{eo})E} \tag{2}$$

where $\mu_{ep,NADH}$ is the electrophoretic mobility of NADH. Based upon the experimental electro-osmotic flow of $4.4 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}, an electric field strength of 125 V/cm, and the electrophoretic mobilites given in Table I, the detection window for the assays depicted in Fig. 1 extended from approximately 350 to 740 s.

Reproducibility of EMMA method

Fifteen replicate determinations of a sample containing 0.5 mg/ml ethanol were made to evaluate the assay reproducibility. Quantitation based upon NADH peak area yielded a relative standard deviation (R.S.D.) of 7.0%. However, this lack of precision can be largely attributed to the irreproducibility of the hydrodynamic injection volumes (R.S.D. of injection volume was 7.1% for this study based upon peak area of internal standard). This lack of precision of sample introduction can be ascribed to the fact that we utilized the minimum possible injection volume of the BioFocus 3000's pressure injection system (pressure · time constant of 1 p.s.i. · s corresponding to an injection volume of approximately 3 nl as calculated by the Poiseuille equation). Because precision in capillary electrophoresis is largely dependent upon the reproducibility of sample introduction [9], an internal standard was used to compensate for error in injection. p-Nitrophenol was selected as the internal standard for this study because it exhibited high absorbance at 340 nm at alkaline pH and was sufficiently anionic at pH 9 to prevent comigration with the NADH peak. When quantitation was based upon the ratio of NADH peak area to p-nitrophenol peak area, R.S.D. for this study improved to 3.0%. Fig. 2 illustrates an EMMA determination of ethanol utilizing p-nitrophenol as the internal standard.
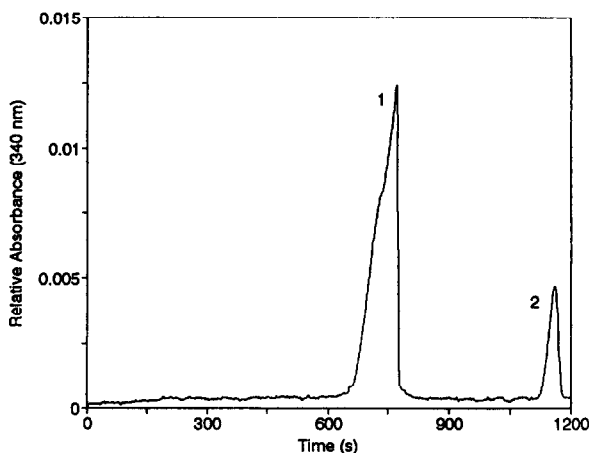
Temporal peak width and, consequently, peak

Fig. 2. EMMA determination of 1 mg/ml sample of ethanol. Peaks: 1 = NADH (analytical reaction product); 2 = p-nitrophenol (internal standard). For conditions see text.

area in capillary electrophoresis are inversely proportional to the instantaneous velocity of the detected species passing the detection window [10]:

$$area \propto \frac{1}{(\mu_{ep,det} + \mu_{eo})E} \tag{3}$$

where $\mu_{ep,det}$ is the electrophoretic mobility of the detected species. Thus, compensating for variability in transport velocity further improved reproducibility in our EMMA determinations of ethanol. Normalization was achieved by dividing each peak area by its migration time. The migration time of the NADH interfacial peak represents the migration velocity of the NADH because reduced cofactor in this peak traverses the entire capillary. NADH formed later in the assay travels only a portion of the length of the capillary. Although this normalization procedure actually utilizes the average migration velocity of the detected species rather than the instantaneous velocity at the detection window, quantitation based upon the ratio of NADH peak area to internal standard peak area, each normalized by dividing by their respective migration times, further reduced the R.S.D. to 2.7%.

Linearity of EMMA method

Fig. 3 depicts a typical calibration curve obtained in the EMMA determination of ethanol
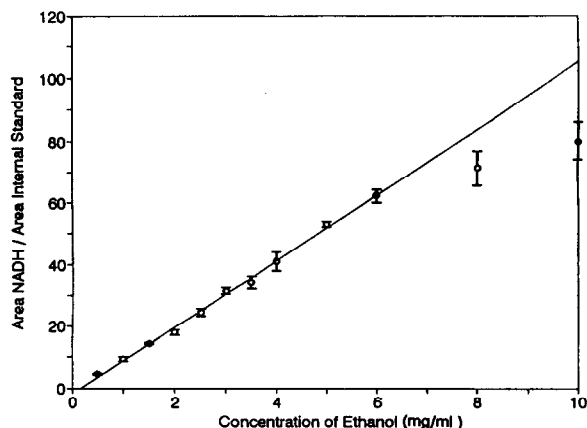
Fig. 3. Calibration curve for EMMA determination of ethanol. Points are the mean of three replicate determinations. Brackets indicate 95% confidence intervals. Line represents linear regression of 0.5 to 6 mg/ml data ($R^2 = 0.997$). For conditions see text.

samples ranging from 0.5 to 10 mg/ml. Each data point represents the mean of three replicate determinations. Quantitation was based upon the ratio of NADH and internal standard peak areas each normalized by their respective migration times. The linear range extended from 0.5 to 6 mg/ml. Linear regression of the 0.5 to 6 mg/ml data yielded $y = 10.72x - 1.844$ with a correlation coefficient of 0.997. Truncation of the NADH profile at the lower limit of the NADH detection window (eqn. 2) was observed beyond 6 mg/ml, thereby causing the response to deviate from linearity because the available reaction time (eqn. 1) was insufficient to fully deplete the ethanol. Less precision was also observed for concentrations which experienced truncation. This phenomenon was due to the fact that the internal standard was not able to compensate for the variable degree of truncation experienced with differing volumes of the same concentration. Linear regression of NADH peak areas without internal standard or migration time normalization produced a correlation coefficient of 0.969 for the 0.5 to 6 mg/ml data depicted in Fig. 3.

The linear range of the EMMA assay can be extended by increasing either the substrate incubation time or the rate of reaction. The available reaction time may be increased by decreasing the electric field strength or by increasing the separation length of the capillary (eqn. 1). However,

these methods also result in a concurrent increase in the analysis time that is imposed by the upper limit of the NADH detection window (eqn. 2) or by the increased migration time of the internal standard if one is utilized. The linear range was extended without adversely affecting the analysis time by elevating the concentrations of the analytical reagents, thereby increasing the rate of reaction. Fig. 4 illustrates the effect of ADH concentration in the analytical reagent/running buffer on the upper limit of the linear range.

Comparison of EMMA to spectrophotometric method

An inter-method correlation study confirmed the validity of the EMMA technique. Eight samples contained between 0 and 4 mg/ml of ethanol were analyzed by both the EMMA procedure and the Sigma Diagnostics Alcohol (Ethanol) kit. The Sigma spectrophotometric method employs the same enzymatic system as the EMMA determination. Fig. 5 compares the results of the EMMA (x) and Sigma (y) methods. Linear regression analysis of the data yielded $y = 1.04x - 0.038$ mg/ml, with a correlation coefficient of 0.995. The paired results of the eight samples produced a paired Student's t calculation of 0.284 compared to a table value ($\alpha = 0.05$; 95% confidence interval) of 2.365. These results indicated that the two methods did
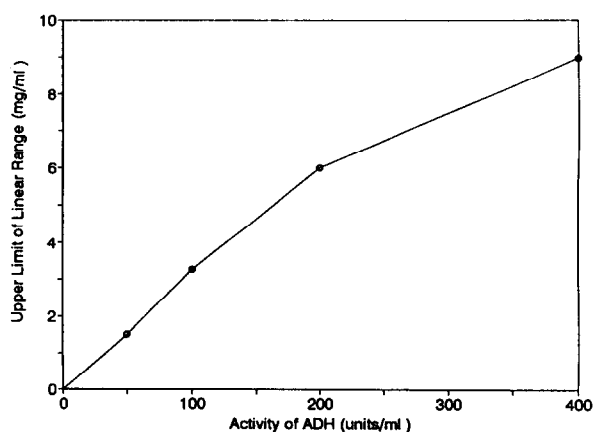

Fig. 4. Effect of concentration of alcohol dehydrogenase in analytical reagent/running buffer on the upper limit of the linear range for EMMA determination of ethanol. For conditions see text.

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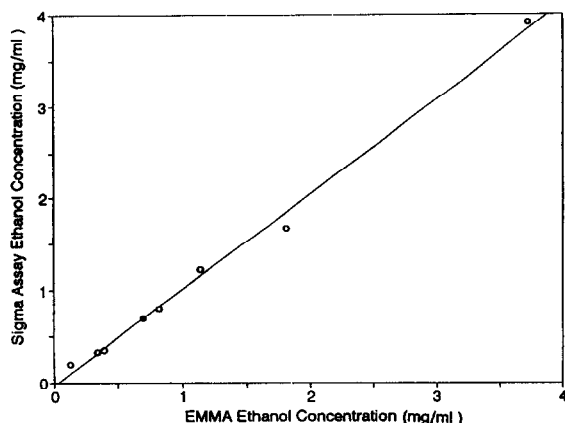B.J. Harmon et al. / J. Chromatogr. A 657 (1993) 429–434

Fig. 5. Correlation of eight ethanol determinations by EMMA and Sigma spectrophotometric methods. Line represents linear regression results ($R^2 = 0.995$). Conditions stated in text.

not yield significantly different values for the determination of ethanol.

Advantages of EMMA methodology

The advantages of the EMMA methodology include those of electrophoretic mixing and miniaturization. As chemical species electrophorese essentially independently of the bulk solution, analytes can encounter many times their own volume in analytical reagents without experiencing the dilution associated with bulk mixing. In the EMMA determinations depicted in Fig. 1, the ethanol zone encountered approximately 46 and 35 times its own initial volume in ADH and NAD^+, respectively, based upon the 3 nl initial volume of the analyte plug, the differential mobilities of the reagents, and the separation length of the capillary.

The EMMA technique also requires minimal volumes of analyte. The assays shown in Fig. 1 were performed on an injection of approximately 3 nl of sample. However, the logistics of sample introduction in current capillary electrophoresis systems generally require several μl of sample actually be available. The lower limit of detection by UV absorption for the EMMA method was approximately 4 μg/ml of ethanol (300 fmol based upon an injection volume of 3 nl). However, this detection limit could be lowered by two orders of magnitude by the use of laser-induced fluorescence detection of NADH. The

detection limits for EMMA determinations of substrates do not approach those reported in the analysis of enzymes [1,3] due to the non-amplifying nature of the assay. In addition to the potential for determining ultramicro samples, the EMMA method consumes very small amounts of analytical reagents. For the 13-min assays depicted in Fig. 1, only 0.8 μl of analytical reagent/ running buffer solution was depleted due to the bulk electroosmotic flow.

We have performed the EMMA ethanol assay without sample preparation on beverage samples and on ultrafiltrate samples obtained from the interstitial fluid of rats dosed with ethanol. No significant interferences were observed. However, the determination of blood samples does require sample preparation (*i.e.* deproteinization) as adsorption of matrix components to the capillary's silica surface greatly diminishes reproducibility. We have not yet attempted to perform the EMMA ethanol determination on surface-modified capillaries as a method to prevent this phenomenon.

ACKNOWLEDGEMENTS

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