

## Evaluation of a Modified Alcohol Dehydrogenase Assay for the Determination of Ethanol in Blood

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We evaluated a new alcohol dehydrogenase (EC 1.1.1.1) enzymic assay (ADH-glycine, Sigma Chemical Co.) for the determination of ethanol in blood. This assay differs from the manufacturer's previous assay (ADH-pyrophosphate) in that glycine replaces pyrophosphate as the buffer and hydrazine replaces semicarbazide as the trapping agent. The standard curve for the assay was linear over blood ethanol concentrations of 0.50–5.00 g/L. The reaction time of the assay was 10 min. At 1.00 g/L within-run and between-run CVs were 3.96% ( $n = 20$ ) and 4.01% ( $n = 20$ ), respectively. Mean analytical recovery of ethanol added to whole blood at 0.50–5.00 g/L was 99.7% (SD 2.6%). We performed 100 consecutive clinical and forensic determinations by the ADH-glycine assay, the ADH-pyrophosphate assay, and gas chromatography. Correlation coefficients of the results by least-squares linear regression were 0.995 for ADH-pyrophosphate vs ADH-glycine, and 0.990 for gas chromatography vs ADH-glycine. The major advantage of the ADH-glycine assay over the ADH-pyrophosphate assay is the shorter reaction time, 10 min vs 30 min.

**Additional Keyphrases:** *forensic medicine • enzymic methods*

The alcohol dehydrogenase (ADH, EC 1.1.1.1) method (1) is commonly used for ethanol determinations in clinical laboratories. We evaluated a new ADH assay (332-UV; Sigma Chemical Co., St. Louis, MO 63178) for the determination of ethanol in blood. The new assay (ADH-glycine) differs from this manufacturer's previous assay (331-UV) in that glycine has replaced pyrophosphate as the buffer and hydrazine has replaced semicarbazide as the trapping agent. We determined the linear range, reaction time, precision, accuracy, and specificity of the new assay and compared the results of 100 consecutive clinical and forensic blood determinations by the new assay, the previous assay, and gas chromatography.

### Materials and Methods

#### Apparatus

**ADH analysis.** All ADH analyses were performed with a Stasar III spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH 44074) equipped with a thermal control unit set at  $25 \text{ }^\circ\text{C} \pm 0.1 \text{ }^\circ\text{C}$  and interfaced with a CP-5000 programmer-data processing unit (Syva Co., Palo Alto, CA 94303).

**Gas-chromatographic analysis.** For gas chromatography, we used a Varian Model 2400 gas-chromatograph (Varian

Aerograph, Palo Alto, CA 94303), recording the flame-ionization detector response with a 1-mV potentiometric strip-chart recorder (Model 023; Perkin-Elmer Corp., Norwalk, CT 06856).

#### Reagents

All reagents necessary for ADH blood ethanol determinations were obtained from Sigma Chemical Co.: ADH-NAD Multiple Test Vial, cat. no. 331-30 (ADH-pyrophosphate), containing 20  $\mu\text{mol}$  of  $\text{NAD}^+$  and 1650 U of ADH; ADH-NAD Multiple Test Vial, cat. no. 332-5 (ADH-glycine), containing 9  $\mu\text{mol}$  of  $\text{NAD}^+$  and 750 U of ADH; glycine buffer reagent, 0.3 mol/L, pH 9, cat. no. 332-9; pyrophosphate buffer solution, 0.075 mol/L, pH 9, cat. no. 330-30; and trichloroacetic acid solution, 6.25 g/L, cat. no. 331-7. All other reagents were AR grade.

#### Standards

Absolute ethanol was diluted with distilled water to produce aqueous working standards containing 0.50, 0.75, 1.00, 1.50, 2.00, 3.00, and 5.00 g of ethanol per liter. Commercial aqueous ethanol standards, 0.80 g/L (Sigma) and 1.50 g/L (College of American Pathologists, Skokie, IL 60077) were analyzed with each group of blood specimens as quality-control samples. All standards were stored at  $4 \text{ }^\circ\text{C}$  in borosilicate glass containers.

#### Procedures

**ADH-glycine assay.** Prepare a protein-free supernate of blood by pipetting 0.50 mL of whole blood into 2.0 mL of trichloroacetic acid reagent. Cap the mixture immediately, mix by inverting several times, and centrifuge for 5 min at 3000 rpm. Prepare the ADH assay enzyme by adding 16.0 mL of glycine buffer reagent to the ADH-NAD Multiple Test Vial. Add 50  $\mu\text{L}$  of protein-free supernate to 3.0 mL of ADH-NAD/glycine buffer. Immediately cap the reaction mixture, mix by inversion, and allow it to stand at room temperature for 5–10 min to allow the reaction to run to completion. Treat an aqueous blank, the ethanol standards, and the quality-control samples in the same manner as whole-blood specimens. Read the absorbance at 340 nm of the whole-blood test specimens and standard solutions vs the aqueous blank as reference, and record with a spectrometer/data-processing unit. All specimens are analyzed in duplicate. Determine the concentration of ethanol in the blood specimens from the calibration curve of the aqueous standards.

**ADH-pyrophosphate assay.** The ADH-pyrophosphate assay procedure is the same as ADH-glycine assay except that Sigma 332-5 replaces Sigma 331-30 as the ADH-NAD Multiple Test Vial, pyrophosphate replaces glycine as the buffer solution, and the capped reaction mixture is allowed to stand for 30 min.

**Gas chromatography.** These analyses on aqueous standards and whole blood were done by the direct injection method as described by Dubowski (2). Briefly, the procedure involves mixing equal volumes of whole blood and internal standard

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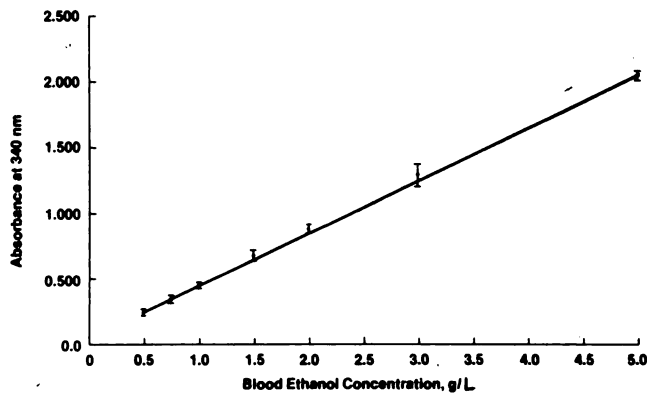


Fig. 1. Calibration curve for blood ethanol determinations by ADH-glycine assay  
Points = mean of three determinations, 2 SD in brackets

(acetonitrile), centrifuging, and injecting 2  $\mu$ L of clear supernate into the gas chromatograph. Samples were analyzed in duplicate. The detector responses were recorded on a 250-mm-wide chart and the concentration of test samples were calculated by comparison with the the reference samples by peak-height measurements. Samples were chromatographed in a 1.8 m  $\times$  4 mm (i.d.) stainless-steel column packed with Porapak S, 80–100 mesh (Waters Associates, Inc., Milford, MA 01757). Typical operating conditions were: nitrogen (carrier gas) flow, 45 mL/min at 55 psi [1 psi  $\approx$  6.9 kPa]; hydrogen flow, 45 mL/min at 45 psi; air flow, 330 mL/min, at 150 psi; column temperature 165  $^{\circ}$ C; injection port temperature, 175  $^{\circ}$ C; and detector temperature, 225  $^{\circ}$ C. The retention times in minutes for various alcohols were: methanol 1.0; ethanol, 2.0; acetonitrile (internal standard), 1.5; and isopropanol, 3.6.

## Results

The time required for complete reaction of ethanol with the ADH-glycine assay was 10 min at 25  $^{\circ}$ C over an ethanol concentration range of 0.50–5.00 g/L. Figure 1 shows a typical calibration curve obtained with the assay over this concentration range. The calibration curve corresponded to the least-squares linear regression equation:  $y = 0.004x + 0.05$  ( $r = 0.998$ ). The manufacturer recommends use of 0.10 mL of protein-free filtrate in the assay and indicates a linear range of 0.50–3.00 g of ethanol per liter. A calibration curve prepared by ethanol standards in that range corresponded to the least-squares linear regression equation  $y = 0.004x + 0.02$  ( $r = 0.996$ ). By using only 50  $\mu$ L of protein-free filtrate we could extend the linear range to 5.00 g/L.

The within-run and between-run precision (CV) of the assay for a control specimen of whole blood with a target ethanol value of 1.00 g/L was 3.96% ( $1.026 \pm 0.041$  g/L, mean  $\pm$  SD,  $n = 20$ ) and 4.01% ( $1.033 \pm 0.041$  g/L,  $n = 20$ ), respectively. The latter represents data for runs on five successive days. The between-run CV for the 1.50 g/L aqueous ethanol quality-control sample over 20 weeks was 1.57%. Recovery experiments with 0.50, 1.00, 1.50, 2.00, 3.00, and 5.00 g of added ethanol per liter of specimen yielded the following results (mean  $\pm$  SD,  $n = 5$ ):  $0.52 \pm 0.012$ ,  $1.00 \pm 0.035$ ,  $1.47 \pm 0.050$ ,  $2.03 \pm 0.075$ ,  $2.87 \pm 0.029$ , and  $4.95 \pm 0.092$  g/L, for corresponding mean recoveries of 103, 100, 98, 102, 96, and 99%. The overall mean recovery was 99.7%.

We performed an inter-method comparison study on 100 consecutive clinical and forensic blood specimens containing ethanol by analyzing each specimen by the new assay (ADH-glycine), the previous assay (ADH-pyrophosphate), and gas chromatography. Figure 2 compares the results of the

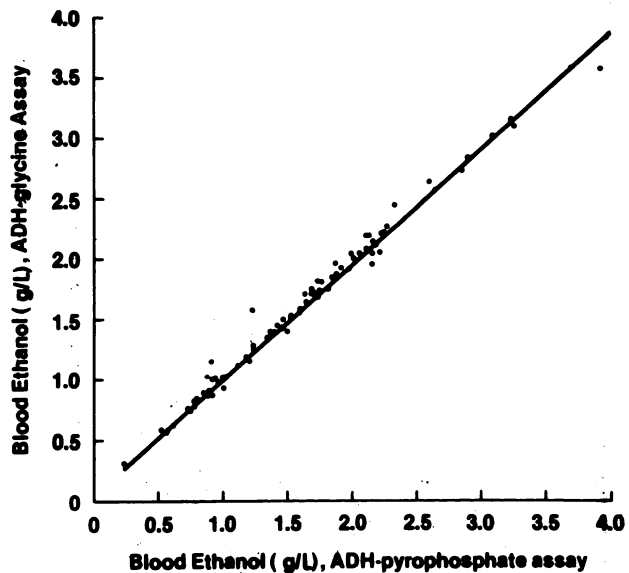


Fig. 2. Correlation of the results of 100 blood ethanol determinations by ADH-pyrophosphate assay and the present ADH-glycine assay ( $r = 0.995$ )

ADH-pyrophosphate assay ( $x$ ) and ADH-glycine assay ( $y$ ). Least-squares linear regression analysis of these data yielded  $y = 0.957x + 4.52$  g/L, with  $r = 0.995$ ,  $\bar{x} = 1.585$  g/L, and  $\bar{y} = 1.561$  g/L. The standard error of estimate ( $S_{y \cdot x}$ ) was 0.044 g/L. Figure 3 compares the results of the gas-chromatographic analysis ( $x$ ) and the ADH-glycine assay ( $y$ ). Least-square linear regression analysis of these data yielded  $y = 1.030x - 5.84$  g/L, with  $r = 0.990$ ,  $S_{y \cdot x} = 0.090$  g/L,  $\bar{x} = 1.572$  g/L, and  $\bar{y} = 1.561$  g/L. The mean difference in the results for the blood ethanol concentrations obtained by the gas-chromatographic and ADH-glycine procedures ( $x - y$ ) was 0.011 g/L for all 100 specimen pairs; 54 of the ( $x - y$ ) differences were positive, 44 negative. The paired results of the 100 specimens yielded paired- $t$  calculations of  $t_{\text{CALC}} = 1.096$  with a table value  $t_{0.950} = 1.984$ .

The specificity of the assay was determined by adding 1.50 g of acetone, ethylene glycol, isopropanol, or methanol per liter of blood and performing ADH-glycine analyses of the speci-

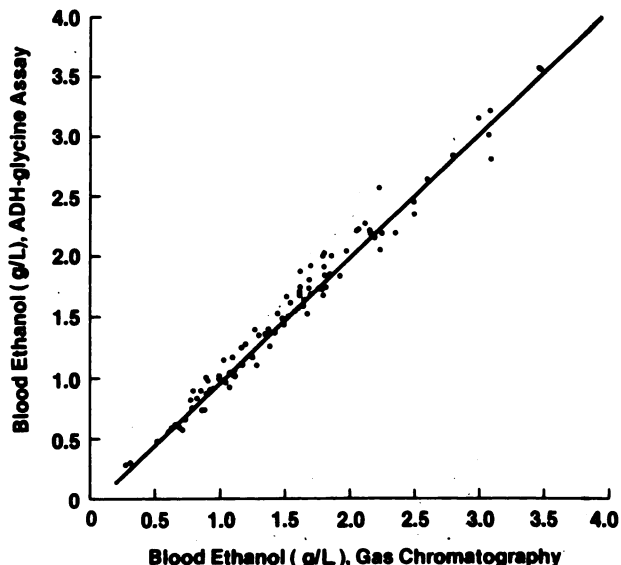


Fig. 3. Correlation of the results of 100 blood ethanol determinations by gas chromatography and the present ADH-glycine assay ( $r = 0.990$ )

mens. Acetone and methanol failed to react; however, ethylene glycol (4.00 g/L) and isopropanol (1.50 g/L) produced false-positive reactions in 10 min equivalent to 0.40 g of ethanol per liter.

## Discussion

The major advantage of the new ADH-glycine assay over the manufacturer's previous ADH-pyrophosphate assay is the shorter time (10 min) required for the reaction to be completed. Our laboratory has routinely performed ADH ethanol determination for 15 years, and during this period we noted that the ADH-pyrophosphate assay often required 30 min for reaction completion. As the pyrophosphate buffer ages, reaction times occasionally increase to 45 min. While we have only six months' experience with new assay, we have not observed any lengthening of the required reaction time.

The major disadvantage of the new assay is that shared by all ADH assays: reaction with alcohols other than ethanol (3, 4). The rate of oxidation of these alcohols decreases in the following order: ethanol = allyl alcohol > *n*-propanol > *n*-butanol > *n*-amyl alcohol > isopropanol (5). In our experience, isopropanol is the only reacting alcohol, other than ethanol, that may occasionally be encountered clinically. Gas-chromatographic analysis of blood, before ADH determination of ethanol, would indicate the presence of isopropanol or other volatile substances (6). Acetone, methanol, *sec*-butyl, and isobutyl alcohol are not oxidized by ADH.

The new ADH-glycine assay gave precise and accurate measurement of blood ethanol concentrations. The results of the new assay compare well with those obtained by the previous ADH assay and by gas chromatography.

We thank Sigma Chemical Co., St. Louis, MO, for generously supplying the ADH reagents necessary for this study.

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# Simultaneous Liquid-Chromatographic Determination of Carbamazepine and Its Epoxide Metabolite in Plasma

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We describe a liquid-chromatographic method for the simultaneous quantification of carbamazepine and its 10,11-epoxide metabolite in plasma. The method is used routinely in the analysis of carbamazepine and its epoxide in 0.5 mL of plasma at concentrations of 1 to 20 and 0.2 to 5 mg/L, respectively. The use of peak-height ratios as a measure of detector response appeared to provide better precision and accuracy than peak-area ratios.

**Additional Keyphrases:** drug assay • anticonvulsants • epilepsy • standard-addition method • therapeutic concentrations

Carbamazepine (CBZ), an effective anticonvulsant commonly used in the treatment of grand mal and psychomotor epilepsy, is also the drug of choice in trigeminal neuralgia. In studies demonstrating a relationship between serum or plasma concentration of carbamazepine and degree of seizure control

(1-5), in general the therapeutic range appears to be 4 to 10 mg/L. The 10,11-epoxide metabolite (CBZE), however, also appears to be an active anticonvulsant (6), and variation in therapeutic response to a given concentration of CBZ in serum may be explained in part by the presence of steady-state concentrations of the epoxide metabolite (7).

Several methods of analysis for CBZ in plasma have been described: spectrophotometry, fluorometry, gas-liquid chromatography, "high-pressure" liquid chromatography (8-10). Methods have also been reported for the simultaneous measurement of CBZ and CBZE in human plasma. A previously reported (11) liquid-chromatographic method for CBZ and CBZE appears to be adequately sensitive for CBZ but, because of interference from an endogenous compound that co-elutes with CBZE, its sensitivity for the metabolite is somewhat limited. Analysis for CBZ and CBZE by a method involving precipitation of plasma proteins is not suitable because phenytoin, an anticonvulsant that is commonly used with CBZ, interferes (12).

The observation that CBZE may contribute to the anticonvulsant effect of the parent drug suggests that monitoring both CBZ and CBZE may be of value. In addition, if the induction of CBZ caused by its prior administration occurs via the epoxide pathway, the CBZE/CBZ ratio during chronic

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