Serum-Ethanol Determination: Comparison of Lactate and Lactate Dehydrogenase Interference in Three Enzymatic Assays

Jeffrey S. Nine, Michael Moraca, Mohamed A. Virji, and **Kalipatnapu N. Rao***

Department of Pathology, Division of Clinical Chemistry, University of Pittsburgh Medical Center, 200 Lothrop Street, Pittsburgh, Pennsylvania

Abstract

Gas chromatography is considered to be the reference method for ethyl alcohol determination. However, enzymatic ethanol assays have been developed for use in the clinical laboratory by several commercial vendors. Essentially, these assays utilize the oxidation of ethyl alcohol to acetaldehyde with concurrent reduction of nicotinamide adenine dinucleotide (NAD) to NADH while monitoring the increase in absorbance at 340 nm. The increase in absorbance is theoretically proportional to the ethanol concentration in the sample. Previously, several authors reported that increased concentrations of lactate and lactate dehydrogenase (LDH) can cause false-positive results with certain enzymatic ethyl alcohol assays. In the present investigation, we further studied the interference of lactate and LDH in three enzymatic assays. Apparent ethyl alcohol concentrations in serum spiked with lactate and LDH, as well as patient and autopsy samples, were determined by the Syva, Abbott, and Roche enzymatic assays and by gas chromatography. The effect of coenzyme depletion on the rate of reaction and the interference of hemolysis were also investigated. Based on our results we suggest that coenzyme depletion plays a major role in the severity of the false-positive ethyl alcohol result, and the interference from hemolysis has a negligible effect on these results. We also confirm the previous studies in showing that elevated serum-lactate and LDH concentrations can result in varying degrees of false-positive ethyl alcohol concentrations in the three enzymatic assays. This should be taken into consideration in the management of patients in a tertiary care medical center.

Introduction

Ethyl alcohol (ethanol) determination is one of the most common toxicology tests performed in clinical chemistry laboratories and is one with many potential medicolegal ramifications. Gas chromatography is the reference method for ethanol determination (1), but it is too costly and time-consuming to be utilized as a primary screening assay. However, rapid enzymatic ethanol assays for use in clinical laboratories have been developed. These assays utilize the oxidation of ethanol to acetaldehyde by alcohol dehydrogenase, with the concurrent reduction of nicotinamide adenine dinucleotide (NAD) to NADH (2,3) to generate ethanol results. We perform approximately 250 serum-ethanol determinations per month using the Syva EMIT (Syva Company, Palo Alto, CA) ethyl alcohol assay on a Cobas Mira S instrument.

We encountered two cases of apparent false-positive ethanol values from our pediatric population. The first case involved a 4-month old boy (28-week gestation) with extensive postnatal respiratory problems. He was found in cardiopulmonary arrest following an upper respiratory tract infection. Resuscitation included bicarbonate, lidocaine, atropine, and epinephrine administration. A serum sample was obtained in the emergency department. Enzymatic ethanol determination was 105 mg/dL. No ethanol was detected by headspace gas chromatography.

The second case involved a 2-month old, full-term girl, born to a mother with a history of alcohol, cocaine, and tobacco abuse. The infant was found in full cardiopulmonary arrest secondary to accidental asphyxiation while sleeping. Resuscitation included bicarbonate, atropine, and epinephrine administration. A serum sample was obtained in the emergency department. Enzymatic ethanol determination was 60 mg/dL. No ethanol was detected by headspace gas chromatography.

The ethanol results in these two cases led to a reevaluation of the Syva ethanol assay procedure. Investigation for the source of interference began with an examination of the reaction mechanism used in the Syva assay (4). Alcohol dehydrogenase (ADH) oxidizes ethyl alcohol to acetaldehyde using the coenzyme NAD, which is concurrently reduced to form NADH:

$$\text{ethyl alcohol} + \text{NAD} \overset{\text{ADH}}{\leftrightarrows} \text{acetaldehyde} + \text{NADH}$$

The increase of absorbance at 340 nm due to NADH formation is proportional to the ethanol concentration in the sample. Therefore, other enzymatic reactions that use NAD as a coenzyme and generate NADH could interfere in the ethanol assay. Based on the findings from recent reports of false-

* Address correspondence to Dr. K.N. Rao, Clinical Chemistry-CLSI, Room 5835 Main Tower, University of Pittsburgh Medical Center, 200 Lothrop Street, Pittsburgh, PA 15213-2582.

positive ethanol screens (5,6), we investigated the interference of lactate and lactate dehydrogenase (LDH) on the three enzymatic methods under consideration for ethanol determination in our laboratory. The three assays evaluated were the current assay (Syva), the Abbott REA ethanol assay (Abbott Laboratories, North Chicago, IL), and the Roche ethanol assay (Roche Diagnostic Systems, Nutley, NJ). Headspace gas chromatography was used as the reference method.

Materials and Methods

Headspace gas chromatography. Ethanol analysis was performed on a Perkin-Elmer 8500 gas chromatograph equipped with an HS-6 headspace sampler and a flame-ionization detector; the procedure outlined by Sutheimer et al. (7) was used. A 6-ft. × 2-mm i.d. glass column packed with 5% Carbowax 20M on 60–80 mesh Carbopak B (Perkin-Elmer) was used. The oven temperature was 70°C, carrier gas (helium) flow was 25 mL/min, injector temperature was 150°C, and detector temperature was 250°C. The sample incubation time was 15 min at 60°C. A six-point standard curve (aqueous standards prepared in the laboratory) determined the relationship between peak height and ethanol concentration. The assay is linear to 320 mg/dL and has a limit of quantitation (LOQ) of 1 mg/dL. Bio-Rad trilevel serum-ethanol controls were used for assay validation. *n*-Propanol was used as the internal standard.

Syva. Syva EMIT ethyl alcohol assay reagents were used on the Cobas Mira S according to the instrument parameters provided by Syva. The assay, which employs a one-point calibration (Syva 100-mg/dL calibrator), is linear to 600 mg/dL and has an LOQ of 10 mg/dL (4). Bio-Rad trilevel serum-ethanol controls were used for assay validation. No sample pretreatment was required.

Roche. Roche reagents for ethanol were used on the Cobas Mira S according to the instrument parameters provided by Roche. This assay, which also employs a one-point calibration (Syva 100-mg/dL calibrator), is linear to 400 mg/dL and has an LOQ of 1 mg/dL (8). Bio-Rad trilevel serum-ethanol controls were used for assay validation. No sample pretreatment was required.

Abbott. TDx REA ethanol reagents were used on the Abbott TDx analyzer according to the instrument parameters provided by Abbott. The assay utilizes the linked enzyme-catalytic reactions of the oxidation of ethanol to acetaldehyde by alcohol dehydrogenase with concurrent reduction of the coenzyme NAD to NADH, followed by an additional reaction in which the NADH produced is oxidized to NAD in the presence of monotetrazolium dye by diaphorase to form MT-formazan (a fluorescence attenuator). The fluorescence intensity is measured before and after the generation of the MT-formazan using the radiative energy attenuation (REA) technique (9–11). A six-point standard curve (Abbott ethanol calibrators) determines the relationship between fluorescence intensity and ethanol concentration. Abbott trilevel ethanol controls were used for assay validation. The assay is linear to 300 mg/dL and has an LOQ of 10 mg/dL. No sample pretreatment was required.

Serum samples were obtained from 17 autopsy subjects and from two hospital inpatients, and the levels of lactate and LDH were determined.

Serum was also obtained from a healthy volunteer. Lactate and LDH determinations of this sample were performed, and resultant lactate and LDH concentrations were 1.5mM and 113 international units (IU)/L, respectively. There was no ethanol detectable by headspace gas chromatography. Increasing concentrations of lactate (stock solution of 3.3mM, Sigma Catalog number L 1875) and LDH from beef heart (stock solution of 1000 IU/mL, Sigma Catalog number 826-6) were added to the blank serum to obtain known standard concentrations of lactate and LDH. These values were verified on the Kodak Ektachem chemistry analyzer (Eastman Kodak Company, Rochester, NY).

The apparent ethanol content of each patient and spiked sample in this study was determined by the Syva, Abbott, and Roche enzymatic methods and by headspace gas chromatography.

A false-positive ethanol concentration was defined as an ethanol result greater than 10 mg/dL by the Syva and Abbott assays and greater than 1 mg/dL by the Roche assay, for samples in which the ethanol was undetectable by headspace gas chromatography.

Other analytes. The concentration of lactate and the activity of L-lactate–NAD^+ oxidoreductase (EC 1.1.1.27, LDH) were determined by the slide methodology on the Kodak Ektachem 700 chemistry analyzer. Hemoglobin concentrations were determined on hemolysate obtained after pretreatment of red blood cells with 0.1M phosphate buffer solution, pH 7.2, and sonication. Oxyhemoglobin was measured at 415 nm and at 380 nm, and background correction was obtained at 450 nm.

Results

The age, diagnosis, serum-lactate concentration, enzyme activity, and ethanol results by four different assays of 17 autopsy subjects and two hospital inpatients are given in Table I. On comparison of the enzymatic ethanol assay results with those of the gas chromatographic reference method, 15 of the autopsy samples and both inpatient samples showed false-positive serum-ethanol results by the Syva assay. There were three false-positive ethanol results by the Abbott assay and none by the Roche assay. The false-positive ethanol concentrations show a correlation with the elevated lactate and LDH levels.

Sigma stock solutions of LDH and lactate, when analyzed separately for ethanol content, were negative. These stock solutions were then added to the ethanol-free serum to give the samples varying levels of combined lactate and LDH. These levels were then verified by assay on the Kodak Ektachem 700. The spiked samples were then assayed by the three enzymatic methods for apparent ethanol concentration. The results are presented in Table II. All three methods gave false-positive ethanol values to varying levels of combined lactate and LDH. The threshold levels for generating false-positive ethanol results appear to be 682 IU/L LDH and 14mM lactate for the Syva assay,

26,339 IU/L LDH and 26mM lactate for the Abbott assay, and 43,990 IU/L LDH and 6mM lactate for the Roche assay.

A progressive increase in LDH and lactate concentrations seems to cause a disappearance of false-positive ethanol results by the Syva assay (Figure 1). Supplementation of NAD by the addition of 20 µmol NAD to each of the sample test mixtures resulted in the reappearance of false-positive ethanol levels. This confirms that depletion of NAD in the test mixtures of samples containing very high levels of LDH and lactate was responsible for this phenomenon (Figure 2). Because the false-positive ethanol concentrations by the Abbott and Roche assays are quite low, NAD depletion does not appear to be a factor in these assays.

The results presented in Table III suggest that hemolysis represented by hemolysate hemoglobin concentrations from 2167 to 8667 mg/dL does not seem to result in false-positive ethanol concentrations.

Discussion

Ethanol is the most commonly abused drug throughout the world, and analysis of blood-alcohol concentrations forms a significant amount of work for hospital and forensic laboratories. Gas chromatography is the reference method for ethanol determination, as ethanol levels obtained by this method are accurate and precise (1). However, the method is costly and time-consuming and may not be available in clinical laboratories. The development of enzymatic assays based on the catalysis of ethanol to acetaldehyde and on the correlation of the rate of conversion of coenzyme NAD to NADH with ethanol concentration, as well as the assays' availability in kit form for use on automated instruments, has simplified the task of ethanol determination. These assays, as exemplified by the Syva (4), Abbott (9,10), and Roche (8) enzymatic assays are rapid, sensitive, and cost-effective. However, high serum-lactate and LDH concentrations appear to interfere in some of these assays, resulting in false-positive ethanol values.

In agreement with what was previously reported in the literature (5,6), our results show a correlation between increasing lactate and LDH concentrations and false-positive ethanol results. This finding is most noticeable with the Syva assay and is less prevalent in the Abbott and Roche assays. In addition, there is a minimum threshold of combined lactate and LDH concentration that must be present to produce the false-positive ethanol results. With the Syva assay, this threshold is approximately a lactate concentration of 14mM with a concomitant LDH activity of 682 IU/L. The threshold is much higher with the Abbott and Roche assays. Therefore, the correct combination of lactate and LDH present in a serum sample could result in false-positive ethanol concentrations for all three assays. These thresholds for interference begin with lactate and LDH concentrations that are commonly encoun-

Table I. Age, Clinical Information, Serum-Lactate Concentration, and Enzyme Activity

Samples	Age (yr)	Clinical information	Ethanol by method: GC* (mg/dL)	Abbott (mg/dL)	Roche (mg/dL)	Syva (mg/dL)	LDH† (IU/L)	Lactate (mM)
Autopsy subjects								
A-1	15	Acute lymphoblastic leukemia	<1	<10	<1	<10	112	27.9
A-2	16	Nodular sclerosing Hodgkin's disease	<1	<10	<1	<10	266	20.3
A-3	16	Duchenne muscular dystrophy	<1	<10	<1	40	3066	29.8
A-4	11	Liver transplant (biliary atresia)	<1	<10	<1	47	1971	29.9
A-5	4	Hirschsprung's disease	<1	<10	<1	63	4050	30.0
A-6	20	Chronic myelogenous leukemia	<1	22	<1	66	79,800	27.2
A-7	55	Cardiomyopathy and diabetes	<1	<10	<1	71	2413	22.9
A-8	38	AIDS and chronic hepatitis	<1	<10	<1	78	2574	22.8
A-9	67	Pulmonary disease	<1	<10	<1	84	4302	15.0
A-10	65	Cerebral vascular accident	<1	<10	<1	100	17,930	14.7
A-11	34	Post-liver transplant lymphoproliferative disease	<1	10	<1	107	55,429	44.4
A-12	8	Acute nonlymphocytic leukemia	6	10	3	107	66,434	44.3
A-13	39	Liver transplant secondary to cirrhosis	3	12	5	116	89,670	20.0
A-14	8	Chronic active hepatitis	<1	<10	<1	123	13,067	42.8
A-15	5	Hollow visceral myopathy syndrome	<1	<10	<1	126	14,797	36.2
A-16	10	Liver transplant (biliary atresia)	<1	15	<1	130	49,005	61.4
A-17	1	Glycogen storage disease Type II (Pompe's disease)	<1	<10	<1	138	20,231	35.3
Hospital inpatients								
IN-1	75	Chronic liver failure, severe metabolic acidosis, renal failure	<1	QNS‡	<1	17	2379	15.4
IN-2	55	End-stage liver disease	<1	<10	<1	51	8015	12.3
						Normals	<170	0.7–1.8

* GC = Gas chromatography.
† LDH = Lactate dehydrogenase.
‡ QNS = Quantity not sufficient for analysis.

tered in postmortem blood samples and that may well be seen in samples of living patients with elevated lactate and LDH concentrations due to a variety of clinical conditions (Table I).

The apparent disappearance of interference in ethanol determination in the Syva assay at extremely high levels of LDH and lactate appears to be due to coenzyme (NAD) depletion. The Cobas Mira S instrument takes timed readings of the absorbance at 340 nm. The rate of production of NADH determines the slope of the reaction rate. The instrument measures an initial absorbance reading at time zero, then it measures the addition of Reagent 1 (Tris buffer and stabilizers) at 5 s, followed by Reagent 2 (NAD and ADH) at approximately 25 s into the run, resulting in the initiation of the reaction. At this point, absorbance readings are taken at 25-s intervals for a total of 13 cycles. The slope of the reaction rate is determined between Cycles 7 and 13, which are 3 and 6 min after initiation of the reaction. The normal reaction kinetics behave linearly throughout the entire analysis, but in samples with high levels of lactate and LDH, the reaction proceeds rapidly to near completion before the seventh cycle begins. Thus, subsequent read-

Table II. Apparent Ethanol Concentrations in Ethanol-Free Serum Spiked with Several Levels of Lactate Dehydrogenase (LDH) and Lactate

No.	LDH (IU/L)	Lactate (mM)	Method* Syva	Roche	Abbott
1	98	4	<10	<1	<10
2	96	6	<10	<1	<10
3	98	14	<10	<1	<10
4	71	26	<10	<1	<10
5	69	39	<10	<1	<10
6	682	4	<10	<1	<10
7	680	6	<10	<1	<10
8	682	14	13	<1	<10
9	655	26	20	<1	<10
10	653	39	27	<1	<10
11	2846	4	19	<1	<10
12	2845	6	28	<1	<10
13	2846	14	45	<1	<10
14	2819	26	65	<1	<10
15	2818	39	86	<1	<10
16	3776	4	24	<1	<10
17	3775	6	34	<1	<10
18	3776	14	54	<1	<10
19	3749	26	75	<1	<10
20	3748	39	95	<1	<10
21	10,331	4	36	<1	<10
22	10,330	6	48	<1	<10
23	10,331	14	70	<1	<10
24	10,304	26	87	<1	<10
25	10,303	39	112	<1	<10
26	26,366	4	20	<1	<10
27	26,365	6	19	<1	<10
28	26,366	14	24	1	<10
29	26,339	26	33	3	13
30	26,338	39	46	5	15
31	39,341	4	<10	<1	<10
32	39,340	6	13	<1	<10
33	39,341	14	15	3	<10
34	39,314	26	17	6	18
35	39,313	39	14	11	23
36	43,991	4	<10	1	<10
37	43,990	6	<10	2	12
38	43,991	14	<10	6	15
39	43,964	26	<10	10	26
40	43,963	39	<10	17	36

* Values are in milligrams per deciliter.

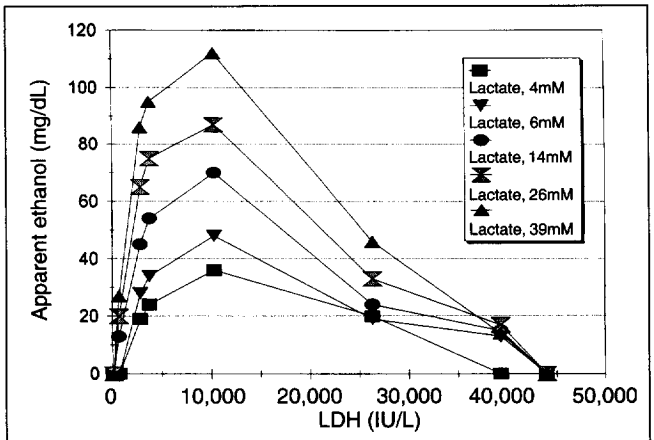

Figure 1. Apparent ethanol concentrations in serum spiked with several concentrations of lactate dehydrogenase (LDH) and lactate by Syva ethanol assay.

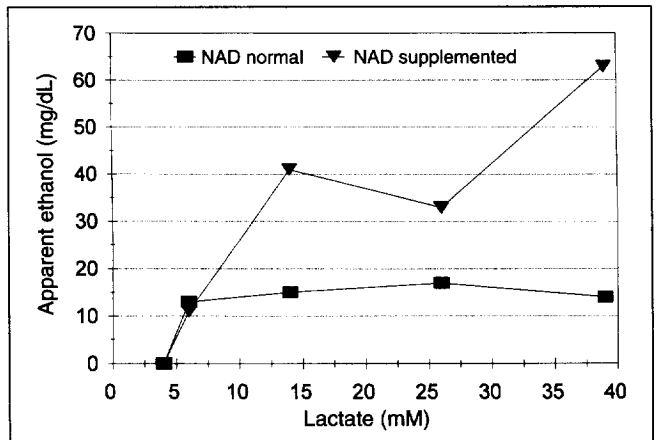

Figure 2. Effect of NAD supplementation on the Syva ethanol assay of serum spiked with lactate dehydrogenase (LDH) and lactate.

Table III. Effect of Hemolysis on Serum-Ethanol Levels by Three Different Methods

Sample	Serum Hb* (mg/dL)	LDH† (IU/L)	Lactate (mM)	Method Syva	Roche	Abbott
1	2167	1372	1	<10	<1	<10
2	4334	2844	2	<10	<1	<10
3	6492	4111	4	11	<1	<10
4	8667	5488	5	11	<1	<10

* HB = Hemoglobin.
† LDH = Lactate dehydrogenase.

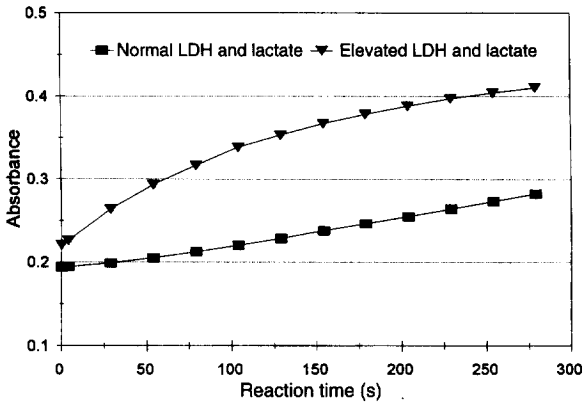

Figure 3. Effect of lactate dehydrogenase (LDH) and lactate on reaction kinetics in Syva ethanol assay.

ings result in a near-zero slope (Figure 3). The instrument interprets this as no reaction and will report a low result or a negative result. The depletion of NAD as the rate-limiting factor in the reaction mixture is confirmed when NAD supplement is added to Reagent 2 and the apparent ethanol concentration of ethanol once again becomes measurable (Figures 1 and 2).

In a medical center with trauma and tertiary clinical care, elevated levels of lactate and LDH occur at a frequency such that these will result in false-positive ethanol results by enzymatic assays. The false-positive ethanol result may have an impact on clinical management in such instances. It is important to ensure that a rapid enzymatic ethanol assay method in use in such a setting is not affected by other constituents in the sample that may participate in the enzymatic reaction.

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