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Determining the Interference of Lactate and Lactate Dehydrogenase in an Ethanol Enzyme Assay

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DETERMINING THE INTERFERENCE OF LACTATE AND LACTATE DEHYDROGENASE IN AN ETHANOL ENZYME ASSAY

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ABSTRACT

Enzymatic ethanol assays are widely used to determine blood alcohol content for individuals admitted to a hospital. In some instances, this hospital data is later used as evidence in a court of law, most commonly in DUI cases. As the enzymatic assay targets, but does not exclusively measure ethanol, it is theoretically possible that interference could occur and produce a falsely elevated result, leading to wrongful convictions or other consequences. This project examined the potential of clinically relevant levels of one potential interferant, lactate and the hepatic enzyme lactate dehydrogenase (LDH) to cause significant ~~and~~ falsely elevated results. Threshold levels of lactate/LDH required and the magnitude of the false positive observed were investigated. False positives were investigated in systems containing no ethanol as well as systems containing high, medium, and low levels of ethanol. These objectives were accomplished by adding varying amounts of lactate, LDH, and ethanol to a simulated hospital assay on a UV-Vis spectrophotometer. The data from this experiment may be applied by forensic toxicologists and pathologists in the interpretation of hospital enzymatic alcohol assay results.

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CHAPTER I

Introduction

Alcohol is a very widely used drug and is therefore present in many clinical and forensic toxicology cases. Quantitation of alcohol levels in patients may affect the clinical treatment they receive as well as any legal consequences associated with the incident that led to their hospitalization. Alcohol analysis and interpretation of its behavioral consequences as well as consideration of the role of the drug in postmortem cases also forms a large part of the forensic toxicologist's workload (Powers & Dean, 2014; Rao, 2012).

Gas chromatography–mass spectrometry (GCMS) or gas chromatography flame ionization detection (GC-FID) is the most accurate and reliable method of determining blood alcohol concentration (BAC) in forensic laboratories. It is, however, expensive, time-consuming, and requires technical proficiency (Powers & Dean, 2014; Rao, 2012). Therefore, BAC determination in a clinical setting is usually accomplished via an alcohol dehydrogenase-based enzymatic ethanol assay, which makes use of the reaction between alcohol and alcohol dehydrogenase (ADH). This reaction has a cofactor, NAD^+, which in the process is reduced to NADH. The level of alcohol in the sample is proportional to the rate at which NADH is produced. NADH absorbs light at 340 nm, (NAD^+ does not) and as such, ultraviolet-visible (UV-Vis) spectroscopy can be used to determine the amount of alcohol present in the sample. This indirect approach can be a concern due to the potential for a competing reaction between lactate and lactate dehydrogenase (LDH) that also produces NADH from NAD^+. Therefore, it is theoretically possible that the presence of lactate and LDH in the serum could give false positive or falsely elevated ethanol concentrations (Powers & Dean, 2009). It is essential that toxicologists understand the levels of lactate and LDH required to have a measurable effect on the assay and the degree to which their presence in serum can change the results, in order to effectively interpret the results of such an assay.

The interference in question has been noticed anecdotally and several studies have been conducted (Badcock & O'Reilly, 1992; Thompson, Malhotra, Schammel, Blackwell, Ward, & Dasgupta, 1994), but only one published study has tested the *in vitro* effect on the assay by spiking serum with lactate and lactate dehydrogenase (Nine, Moraca, Virji, & Rao, 1995). The study used a wide variety in the amounts of lactate and LDH incorporated in the assay, including concentrations far above those reasonably observable in antemortem specimens. Since that study over 20 years ago, the interference of lactate/LDH has been tentatively accepted by the scientific community. However, there is still some contention over its existence, prevalence, and severity; as evidenced by recent articles and court cases contesting its relevance (Powers & Dean, 2009; Nacca, Hodgman, Lao, Elkins, & Holland, 2017). It is problematic that almost every mention of this phenomenon refers back to a single, somewhat limited study in 1995 that failed to adequately resolve the issue (Nine et al., 1995).

Forensically, this research has multiple implications. The presence and level of alcohol in a victim or perpetrator's system can have an influence on their behavior and frame of mind. As a result, the amount of alcohol in a person's blood can serve as a significant piece of evidence in many cases. From a sentencing standpoint, the level of legal consequences assigned to an individual can also be dependent on the blood alcohol concentration (Powers & Dean, 2014).

With these factors in mind, it is imperative that the test used to measure ethanol levels is reliable and accurate, and that any potential interferences or confounding factors be recognized and considered. If there is a possibility that the enzymatic assays used widely throughout hospitals can be inaccurate under certain conditions, it is important that the users and interpreters of those assays know that an interference can occur, and the conditions under which such interferences could have affected an assay.

This project had three aims: to successfully simulate and "scale up" a clinical enzyme assay normally performed on an automatic analyzer for use with a benchtop UV-Visible spectrophotomer, to determine the effect of lactate/lactate dehydrogenase (LDH) added to the assay with no ethanol present, and finally to determine the effect of lactate/LDH added to an assay with varying amounts of ethanol present. This study was limited to amounts of LDH and lactate corresponding to physiologically reasonable levels, both in terms of normal levels, and reasonable elevations that might be encountered in cases of trauma, hypoxia or disease.

Literature Review

Background

Alcohol is a legal and commonly encountered social drug worldwide. Due to its prevalence in American society, numerous laws and restrictions exist to govern its usage (Powers & Dean, 2014). Therefore, analysis and interpretation of an individual's blood alcohol content forms a large part of forensic toxicology casework.

An individual's blood alcohol content (BAC) may be forensically significant in a number of ways. The treatment of an individual when they arrive at an emergency room may be impacted by an underlying BAC (Hubler, Sullivan, & Erickson, 1998; Gharapetian, Holmes, Urquhart, & Rosenburg, 2008). If this treatment is adversely affected by inaccurate test results, medical malpractice suits could ensue. The BAC results of an individual could also come into play as evidence during a trial. For example, the intoxication of a suspect or victim will often be relevant in the course of reconstructing a crime scene or verifying witness statements. At other times intoxication is the main focus of legal action, such as in cases of social host liability (Justice Education Society and Centre for Education, Law, & Society at SFU, 2017), sexual consent, or most commonly, drunk driving.

Alcohol intoxication clearly plays a significant role in legal cases and forensic science. Therefore, it is imperative that tests used to measure it are reliable. As a consequence, forensic laboratories typically use headspace gas chromatography (GC) with mass spectrometry (GCMS) or flame ionization detectors (GC-FID) to accurately and reliably measure BAC (Powers & Dean, 2014). In cases were samples are not submitted to a forensic laboratory, an individual's medical records can also be introduced into legal proceedings, making hospital data potentially relevant as evidence in forensic cases. The use of headspace GC in a clinical setting would be impractical because of its high cost in time, money, and technician hours (Rao, 2014). Instead, hospitals routinely use alcohol dehydrogenase (ADH) based enzymatic ethanol assays to determine BAC.

The alcohol enzymatic assay is based on the rate of metabolism of ethanol present in a sample as catalyzed by ADH. This reaction has a cofactor (NAD^+) which is reduced to NADH in the process. The test usually consists of introducing a sample in a reaction mixture containing ADH, NAD^+, and a buffer (Cheng & Christian, 1978). The rate of appearance of NADH can then be measured at its λ_{max} of 340 nm. Since the initial rate of NADH formation is proportional to the amount of ethanol present, this parameter can be used to calculate the concentration of ethanol in the sample. The sample usually used in this test is serum (to reduce interference from the color of whole blood on absorbance), and the serum concentration is then adjusted using an average ratio to obtain the BAC for whole blood (Powers & Dean, 2014). In contrast to headspace GC, the enzymatic assay is quick and cost-effective (Rao, 2014) and yields quantitatively accurate results a high percentage of the time albeit with a larger underlying procedural uncertainty (Powers & Dean, 2014). From a forensic standpoint, this accuracy is beneficial because hospital data can act as evidence at a later time.

The use of an enzymatic-based alcohol quantification for legal purposes is a concern because it is theoretically possible for the assay to produce positive results from an unintentional co-reaction. The assay indirectly measures the concentration of ethanol via its reaction cofactor; therefore a competing reaction that has the same cofactor could also produce NADH and influence the results (Powers & Dean, 2014). The classic example of a potentially competing reaction is the metabolism of lactate by lactate dehydrogenase, or LDH (Figure 1). This reaction has the potential to cause an interference in the assay by producing NADH independent of ethanol content in the sample (Nine, Moraca, Virji, & Rao, 1995). The presence of NADH generated by the action of LDH on lactate in the test could result in a falsely elevated ethanol concentration result.

In order for lactate/LDH interference to occur, certain conditions must be in place. LDH is an enzyme normally found primarily in the liver, with relatively insignificant concentrations routinely detectable in the blood. High concentrations would only be expected in the blood of individuals with liver damage as a result of chronic disease or trauma (Powers & Dean, 2009). In addition to LDH, the substrate (lactate) must also be present. Lactate is normally seen at low levels in the blood as a metabolic intermediate (Powers & Dean, 2009) but can be found in higher concentrations if the individual has experienced respiratory or metabolic acidosis (Rao, 2014). Treatment with Lactated Ringer's solution has also been suggested as a source of lactate (Rao, 2014). In the presence of high levels of both LDH and lactate, it is theoretically possible to produce NADH independently of ethanol and obtain a falsely elevated result. This interference could thus influence the results without disrupting any of the usual calibrations and quality control measures used to monitor the assay's function (Rao, 2014). This interference is thought to be relatively rare (Powers & Dean, 2014) and has been sparingly studied in recent years.

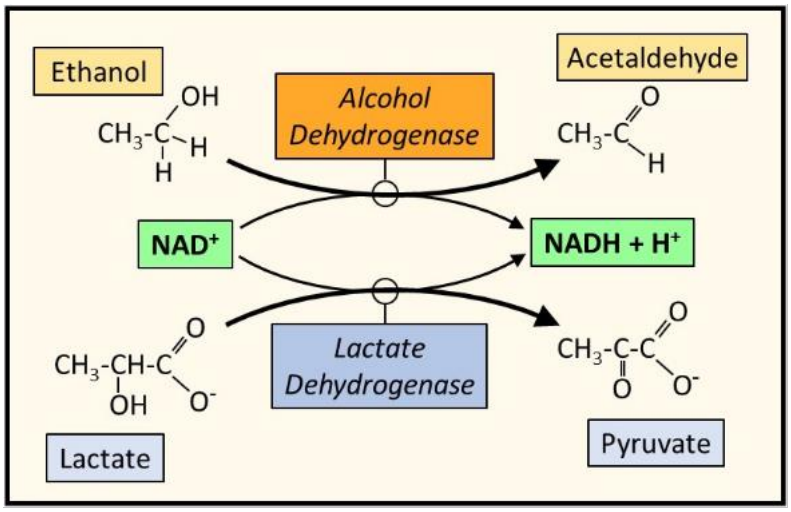

Figure 1: *Mechanism of alcohol metabolism (top) & possible interfering reaction (bottom)[Powers & Dean, 2009; updated 2018]*

Previous Research

The first recognition of the potential for this interference was in 1992, during a study of SIDS (Badcock & O'Reilly, 1992). Researchers noted that postmortem plasma for the infant subjects occasionally tested positive for ethanol when measured with enzymatic assays, but the samples produced negative results when measured with GC. To determine the origin of

the interference, they ran an assay in the absence of ADH and obtained the same positive results. Surmising that lactate/LDH might be the cause, they added lactate and observed an increase in the rate of A340 absorbance increase; then they added an LDH inhibitor and noted a rate decrease. These results led the investigators to conclude that high levels of lactate/LDH in the serum samples could be the cause. In retrospect, this finding was consistent with later published literature, which indicated that concentrations of LDH/lactate increase postmortem (Rao, 2014). This study convincingly established the existence of interference in postmortem cases and provided compelling evidence that high lactate and LDH were responsible.

A few years later, two additional anecdotal cases of living patients were reported, also showing similarly elevated levels of lactate and LDH (Thompson et al., 1994). The researchers noted that this phenomenon could appear in both ante- and post-mortem cases. Further confirmation of the enzymatic basis of the observation was demonstrated by running the tests again after ultrafiltration of the sera, removing protein and hence LDH from the sample. Notably, this step eliminated interference from the assay, suggesting that LDH is somehow involved in the falsely heightened "alcohol" readings, and the interference was not a function of lactate alone. Their study provided more evidence to support the suspected lactate/LDH interference. Thompson et al. (1994) also added varying amounts of lactate and LDH to serum samples to observe the response. This aspect of their study provided more examples of interference but was not systematic enough to define the levels of lactate and LDH in which it might occur.

The most widely cited article on this topic was conducted by Nine et al. (1995), entitled "Serum-Ethanol Determination: Comparison of Lactate and Lactate Dehydrogenase Interference in Three Enzymatic Assays". The study was conducted in two parts. The first part determined the postmortem lactate and LDH levels of 17 autopsy subjects, finding them to be elevated in many cases. The researchers also tested the subjects' ethanol content via

three enzymatic assays (Syva, Roche, and Abbott), with headspace GC-FID used as a reference method. In some of these cases, the elevated lactate/LDH was correlated with false positive ethanol results. This part of the study provided data documenting post-mortem levels of lactate and LDH, albeit mostly for individuals with some underlying disease state.

In the second part of the study, researchers spiked serum from a healthy volunteer with lactate and LDH in various amounts and noted that high levels also caused elevated ethanol readings. This process, however, affected some enzymatic tests more than others. The *in vitro* testing used levels of lactate and LDH far beyond those commonly encountered in clinical antemortem samples, leading to criticism and dismissal of the study by some toxicologists (Winek & Wahba, 1996). This article successfully showed that the interference can happen, including in postmortem cases. This article and associated research had the potential to settle the question of the existence of the interference in antemortem cases using its *in vitro* data but did not successfully do so. Reasons for their failure could be the use of extremely high levels of lactate/LDH, publication in the same article as postmortem studies, or ineffective presentation of data. Regardless, their study did not resolve the issue, as indicated by the conflicting publications since that time.

Despite some criticism, the potential interference of lactate/LDH with the ADH assay has been tentatively accepted in the years since 1995 and is mentioned in toxicology textbooks (Powers & Dean, 2014; Rao, 2012) and courses as a possible but rare occurrence. The issue was again brought to the attention of researchers in 2008, with a letter to the editor communicating 3 more cases of apparently falsely elevated ethanol in a 2-month span (Gharapetian, Holmes, Urquhart, & Rosenburg, 2008). This publication noted that the interference is a problem because it influenced one of the individual's medical care. Another concern raised by the authors was that the levels of lactate and LDH present were not high enough to be a concern according to the threshold values communicated by the enzyme assay

manufacturer. Unfortunately, it is not possible to effectively compare the levels of LDH found in their patients (4871, 8075, and 10,147 U/L) with Nine's study, because their data did not include examples for levels of LDH between 3748 and 10, 331 U/L (Nine, Moraca, Virji, & Rao, 1995).

Soon afterward, the validity of a hospital enzyme assay was questioned in a legal case arising from a DUI charge. In 2009, Powers & Dean published on a court case handled by the Connecticut Department of Public Safety (Powers & Dean, 2009). In the case, a driver had collided with a tree and in the subsequent trial, the hospital records were submitted as evidence of drunk driving. The hospital test resulted in a BAC of 0.17 g/dL, more than twice the legal limit of 0.08 g/dL. The defense challenged the accuracy of the assay on the basis of interference by elevated lactate/LDH. Although the individual's lactate and LDH values were not measured during hospitalization, the authors were able to approximate the amounts of lactate/LDH by using other clinical markers such as the anion gap and levels of hepatic enzyme present in the serum. Using this data, it was concluded that for this particular case the results of the assay were not falsely elevated. However, the authors noted that the interference could possibly be legitimate if the amounts of lactate and LDH were higher. Their article demonstrated that the interference is a relevant concern in the prosecution of modern cases, and further that such cases could be evaluated by examination of clinical parameters.

In 2011, an evaluation of the lactate/LDH interference was conducted by the College of American Pathologists as a part of their proficiency testing program (Frederick & King, 2012). Fifty labs used their normal enzyme assay to test samples containing lactate and LDH in clinically possible concentrations and reported the resulting ethanol concentrations. There was a significant amount of variation depending on the type and brand of instrumentation used; false ethanol readings ranged from 0.004 to 0.106 g/dL. The authors emphasized that this interference is still a problem 20 years after its recognition and offered suggestions for

the testing procedure to eliminate interference. However, the likelihood of these changes being made in clinical settings is low because of their additional effort and cost, and the perceived infrequency of the lactate/LDH based interference. The article showed the prevalence of enzymatic assays and the range of false positives that can occur.

In contrast to most other publications that accepted the potential of interference by lactate/LDH in enzymatic-based alcohol assays, the most recent publication on this topic appeared to dismiss its relevance in a clinical setting. In 2017, a study was published which examined the lactate/LDH levels of live patients presenting to a single hospital over the course of 8 months (Nacca, Hodgman, Lao, Elkins, & Holland, 2017). If the patients had elevated levels of lactate/LDH, their BAC was tested by both enzymatic assay and GC-FID. Results showed only 4 of the 37 patients had a measurable BAC by enzyme assay; all 4 also had similar BACs when confirmed by GC-FID. This led the researchers to state that their data does not support the validity of an interference in live patients.

While this study (as the first conducted on live patients) reflected an important step in the investigation of this issue, the limitations in the experimental design somewhat compromise the overall utility of the research, and limit its applicability to the larger question. The authors themselves recognize that a sample size of 37 is quite small and that their sample included no trauma victims, only victims of disease (Nacca et al., 2017). The lack of trauma victims is a concern because two of the conditions leading to elevated lactate and LDH, hepatic trauma and acidosis, are commonly encountered in trauma victims. It is possible that trauma victims represent a significant number of cases in which this interference occurs. Considering the relative rarity of this phenomenon, it also seems likely that their study was not large enough to catch the occurrences known to occur as indicated in previous publications (Thompson et al., 1994; Frederick & King, 2012; Gharapetian et al., 2008). Another concern is that their study only used a Roche enzyme assay, but claim that their

result “likely applies to the other available products” (Nacca et al., 2017, p.3). Although all enzyme assays operate on similar principles, it is clear from the literature that there is quite a bit of variation from one assay to another (Nine et al., 1995; Frederick & King, 2012). Thus the claim that their results are generalizable is misleading. In fact, the entire study carries risk of misinterpretation. In reality, neither their data nor that which came before provides enough information to confidently disregard or confirm the interference.

Current Research

It is troubling that researchers in this area have only one reference table of interferences and the lactate/LDH levels required to cause them: the data provided by Nine et al., over 20 years ago. Nine’s study made for an excellent beginning, but it is somewhat problematic that the work was not followed by a more definitive study. Scientists working in the field today need an accurate, modern, and complete reference for values of lactate, LDH, and the effect they may have on the ethanol reading from a particular assay. Essentially, thresholds for concentrations of lactate and LDH that can produce a positive result by themselves in an ethanol assay would be of value in most clinical settings, facilitating interpretation of data.

Several issues raised in the Nine study were addressed in this project. First, the levels of lactate and LDH were restricted to clinically reasonable concentrations. Within these parameters, more data points were tested. In particular, the gap between 3748 and 10,331 U/L of LDH, unaddressed by Nine, was investigated. During the course of testing the magnitude of false positives was also determined. In addition to the potential to produce false positives independently, the combinations of lactate and LDH were studied to determine the potential additive effect on existing alcohol levels. This has contributed to a better understanding of situations in which an endogenous level of ethanol is present, but the test results show a higher level of ethanol due to the presence of lactate/LDH.

The aims of this study were to provide a more complete understanding of the levels of lactate and LDH necessary to cause a false positive, the magnitude of the false positive produced, and the effects of high lactate and LDH on a system with endogenous ethanol. The first step was to scale up an enzyme assay for use on a benchtop UV-VIS. The second phase validated, updated, and expanded the data provided by Nine while remaining within concentrations expected in a live patient. The third provided data on the interaction between lactate/LDH along with existing levels of ethanol in that sample. Finally, select samples from Parts II and III were validated using a human serum matrix. Together, these tables of data will be useful as forensic toxicologists and pathologists examine medical records in the course of an investigation. By enriching the information available, the data obtained in this study will allow scientists to more accurately interpret the results of a hospital enzyme assay.

CHAPTER II: MATERIALS & METHODS

Methods

Part I: Standard Curve

The alcohol dehydrogenase enzyme assay was adapted from hospital procedures for use on a Shimadzu UV-1700 absorbance ultraviolet-visible spectrophotometer (UV-Vis). The procedure from SYNCHRON® Systems was used as a template (Beckman Coulter Inc, 2010) to determine initial concentrations and ratios of the assay components. The Beckman Coulter assay is a "continuous flow" assay, which required conversion to a defined volume, single cell assay for use in this project. The assay was then performed on a set of ethanol standard solutions and an ethanol test solution of unknown concentration to determine its accuracy and precision. Adjustments to the assay's reagent concentrations, ratios, and procedures were made. These adjustments included the type and volume of cuvette, instrumental parameters, ratio of assay components to sample, duration of data collection, sample mixing, sample and reagent temperature control, and various measuring devices. These experiments resulted in the following method, used to collect the standard curve data as well as the experimental data in the following sections.

1.1 Solution preparation

All solutions were kept on ice while in use and refrigerated otherwise. Solid enzyme and cofactor powders were kept in the freezer when not in use.

1.1.1 Ethanol standards

140 proof ethanol (Sigma-Aldrich) was serially diluted with distilled water to produce ethanol standards of the concentrations listed in Table 1. 20 mL of each standard was prepared. Class A glass pipets and volumetric flasks were used. These concentrations were verified using gas

TABLE 1: *Concentration of ethanol standards*

Sample No.	Ethanol (mg/dL)
1	0
2	31.25
3	62.5
4	125
5	250
6	500

chromatography with flame ionization detection (GC-FID). The ethanol test solution for determining accuracy/percent error was also prepared using this method; its final concentration was found to be 86 mg/dL, again using GC-FID.

1.1.2 TRIS buffer

~24.2 g Trizma (Sigma-Aldrich) was dissolved in ~900 mL distilled water to produce a 200 mM buffer solution. Concentrated hydrochloric acid was added dropwise to pH ~7.6, then distilled water was added up to 1.0 L.

1.1.3 Nicotinamide adenine dinucleotide (NAD) Stock Solution

~0.12 g of NAD (Sigma-Aldrich) was dissolved in 10 mL TRIS buffer. Solution was prepared fresh each day of use.

1.1.4 Alcohol dehydrogenase (ADH) Stock Solution

~0.001 g of ADH from yeast (Sigma-Aldrich) was dissolved in 10 mL TRIS buffer. Solution was prepared fresh each day of use.

1.1.5 Enzyme mix

500 µL of ADH and 10 mL of NAD stock solutions were combined and diluted 1:5 with buffer to obtain an enzyme/cofactor mix, hereafter referred to as the 'reaction reagent' or 'enzyme mix'. The concentrations of these constituents in the final assay incubation were ~2.5 mM NAD and ~200 U/L ADH.

1.2 Assay Procedures

A triplicate 6-point standard curve was generated for each day of data collection and used to calibrate the results from that day. An additional test solution of ethanol was also analyzed and used to determine the accuracy of the standard curve.

1.2.1 Sample preparation

For each standard, micropipettes were used to deliver 700 µL enzyme mix, 200 µL TRIS buffer, and 100 µL of the ethanol standard into a plastic test tube. All samples had a final incubation volume of 1.0 mL. The contents were mixed by gently 'flicking' the tube three times, then incubated in a room-temperature water bath for 10 seconds. The sample was then transferred to a polystyrene disposable cuvette (pathlength=1 cm) and inserted into the UV-Vis. Data collection was then initiated on the instrument.

1.2.2 Instrument settings

The UV-1700 was operated using Shimadzu UVProbe software in Kinetics mode, to collect the rate of absorbance change at a single wavelength over time. The instrument method collected absorbance at λ=340 nm over a period of 30 seconds, initiated just after insertion of the cuvette. The average rate of absorbance in units of mAbs/min was calculated by the UVProbe software.

1.2.3 Data interpretation

The absorbance rates for each standard were plotted on a substrate-velocity curve and Lineweaver-Burke plot. Lineweaver-Burke plots were produced for each set of standards by plotting the inverse of the concentrations versus the inverse of their average absorbance rates The accuracy and precision of the standards was assessed by calculating percent standard deviation of the triplicate values (acceptance criteria: <20%), trendline fit for the Lineweaver-Burke plot (acceptance criteria: R^2>0.97), and percent error (acceptance criteria: <10%) of the result obtained after running the ethanol positive control solution.

Part II: The Effect of Lactate and LDH on an Assay without Ethanol

In the second phase of the project, the effects of lactate and LDH were studied on the assay containing the reaction reagent but no ethanol.

2.1 Solution preparation

All solutions were kept on ice while in use and refrigerated otherwise. Solid enzyme and cofactor powders were kept in the freezer when not in use. Tris buffer, reaction reagent, and ethanol standards were prepared in the same manner as the previous section.

2.1.1 Lactate solutions

The stock solution (Sigma, ~11,775 mmol/L) was diluted with distilled water serially to produce solutions of the concentrations listed in Table 2. Class A glass pipets and volumetric flasks were used.

TABLE 2
Concentration of lactate samples

Sample No.	Lactate (mmol/L)
1	0
2	1
3	5
4	10
5	25
6	50

2.1.2 LDH Stock solutions

LDH stock solution (Sigma-Aldrich, from rabbit muscle; ~550 U/mg; 10 mg/2 mL) was serially diluted with distilled water to produce solutions of the concentrations listed in Table 3. Class A glass pipets and volumetric flasks were used.

2.1.3 Lactate & LDH solution concentrations

Representative concentrations ranging from normal blood reference levels (lactate 0.5-2.2 mmol/L; LDH 122-225 U/L, Nacca 2017) to the upper limits of clinical concentrations were used (as shown in Tables 2-3).

TABLE 3
Concentration of LDH samples

Sample No.	LDH (U/L)
1	0
2	200
3	1000
4	2000
5	4000
6	6000
7	8000
8	10 000

2.2 Calibration

The assay was calibrated as per section 1.2 above.

2.3 Sample matrices

Using the procedures as noted above (sections 1.12 – 1.23), lactate and LDH were analyzed on the assay in various combinations, resulting in a series of data matrices (see Table 4). In each matrix, the concentration of LDH was held constant while varying the concentration of lactate. Incubation samples included 700 µL reaction reagent, 100 µL lactate, 100 µL LDH, and 100 µL buffer. Each sample was run in triplicate.

2.3.1 Control samples

A matrix of samples with lactate but no LDH was performed to verify that no reaction occurs between lactate and the reaction reagent in the absence of ethanol and LDH. Each sample contained 700 µL of reaction reagent, 200 µL of buffer, and 100 µL of the lactate solution (concentrations listed in Table 2). To verify that no reaction occurs when LDH is added in absence of lactate, a control sample with LDH and reaction reagent, but no ethanol or lactate, was included in each sample matrix.

2.1 Data interpretation

Using the standard curve for the same day, the results were translated into perceived ethanol (mg/dL) results. For each matrix, the results were plotted with perceived BAC in terms of lactate concentration.

TABLE 4: *Example of a sample matrix for Part II*

Reaction reagent	100 µL LDH	100 µL lactate	TRIS buffer
700 µL	8000 U/L	--	200 µL
700 µL	8000 U/L	1.0 mM	100 µL
700 µL	8000 U/L	5.0 mM	100 µL
700 µL	8000 U/L	10.0 mM	100 µL
700 µL	8000 U/L	25.0 mM	100 µL
700 µL	8000 U/L	50.0 mM	100 µL

Part III: The Effect of Lactate and LDH on an Assay with Ethanol Standard Solutions

Part III was similar to Part II, but in these cases the incubation contained ethanol as well as various combinations of lactate and LDH. High (125 mg/dL), medium (62.5 mg/dL) and low (31.25 mg/dL) concentrations of ethanol were added to the matrices from Part II.*3.1 Solution preparation*

Same as Part II.

3.2 Calibration

Same as Part II.

3.3 Sample matrices

Similar procedures to Part II were used, except for the addition of ethanol to each sample. Each matrix (see Table 5) had a constant concentration of LDH and ethanol while varying the concentration of lactate. Incubation samples included 700 µL reaction reagent, 100 µL lactate, 100 µL LDH, and 100 µL ethanol.

TABLE 5: *Example of a sample matrix for Part III*

Reaction reagent	100 µL LDH	100 µL lactate	TRIS buffer	100 µL ethanol
700 µL	8000 U/L	--	100 µL	31.25 mg/dL
700 µL	8000 U/L	1.0 mM	--	31.25 mg/dL
700 µL	8000 U/L	5.0 mM	--	31.25 mg/dL
700 µL	8000 U/L	10.0 mM	--	31.25 mg/dL
700 µL	8000 U/L	25.0 mM	--	31.25 mg/dL
700 µL	8000 U/L	50.0 mM	--	31.25 mg/dL

3.3.1 Control samples

Control samples of lactate and ethanol in the absence of LDH as well as LDH and ethanol in the absence of lactate were conducted as in Part II. In addition, control samples of ethanol with lactate & LDH were performed in absence of ADH to evaluate the effect of ethanol on the reaction between LDH & lactate.

3.4 Data interpretation

Same as Part II.

Part IV: Verification of Results using a Human Serum Matrix

Human serum was used to prepare test solutions and validate the results obtained in buffer for Parts II and III. Similar procedures were followed, except solutions of ethanol, lactate, and LDH were prepared in human serum (Sigma) instead of distilled water.

4.1 Sample matrices

Selected combinations of LDH and lactate were tested with final concentrations of 200, 6000, and 10,000 U/L LDH, and 1, 10, and 50 mM lactate. As above, each matrix held the concentration of LDH constant while varying the amount of lactate. Each sample consisted of 700 µL enzyme mix, 100 µL LDH, 100 µL lactate, and 100µL buffer. Each combination was analyzed in triplicate.

4.2 Data interpretation

The results from the human serum trials were compared with the results from Parts II and III to verify that the results were reasonably similar to serum samples.

Materials

All reagents (Table 6) were procured from Sigma Aldrich, except for those already in stock. Results were gathered from a UV-1700 spectrophotometer (Shimadzu). Digital data was collected using a Dell Latitude E6520 laptop computer, attached to the UV-1700 by a USB-to-RS232 cable. Data reduction was performed by UVProbe 2.60 software (Shimadzu). Standard laboratory glassware and equipment was procured from Fischer Scientific.

TABLE 6: *Materials List*

Reagents	***Equipment***
Ethanol, 140 proof Alcohol dehydrogenase (ADH) from yeast Nicotinamide adenine dinucleotide (NAD) TRIS buffer Lactate Lactate dehydrogenase (LDH) Human serum Hydrochloric acid	UV-1700 (Shimadzu) Reusable cuvettes Reusable test tubes Computer Adapter cable UV Probe 2.6 Software Lab glassware Ice bucket Micropipettes & tips

CHAPTER III: RESULTS & DISCUSSION

Part I: Standard Curve

Ethanol standards used to calibrate the experimental results each day were run in triplicate and evaluated for their accuracy and precision as described in section 1.2, above. There were no instances of standard curve rejection based on failure to meet acceptance

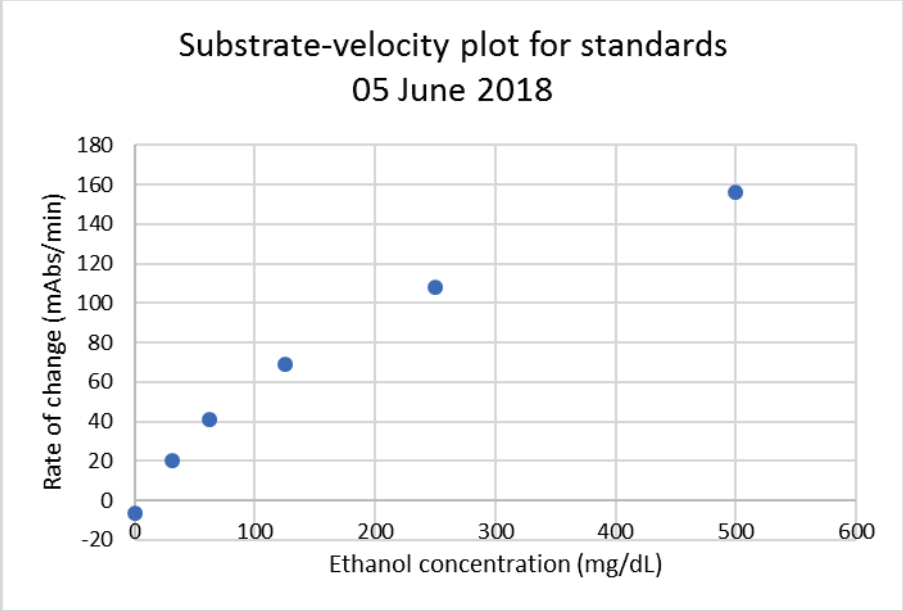

***Figure 2**: Representative example of substrate-velocity plot for ethanol standards*

criteria (Section 1.2.3). The standards were plotted opposite their average rates of absorbance change to obtain a substrate-velocity plot; see Figure 2 for a representative example. The standard curve was evaluated for precision with the use of percent standard deviation of the triplicate calibrators. The percent standard deviations had an average value of 2.5%, and a range of 0.037-15.2% (acceptance criteria: <20%).

Lineweaver-Burke plots were also produced for each set of standards (see Figure 3 for a representative example). The standards were evaluated for accuracy using trendline fit; the best fitting straight line was used as the standard curve for each day of data collection. The R^2 values were typically greater than 0.99; one set of standards had a lower R^2 of 0.978 (acceptance criteria: R^2>0.975). The standard curve was also used to calculate percent error

for the ethanol control test solution (target value 86 mg/dL); these percent errors had an average percent error of 5.35%, with a range of 1-8% error (acceptance criteria: <10%).

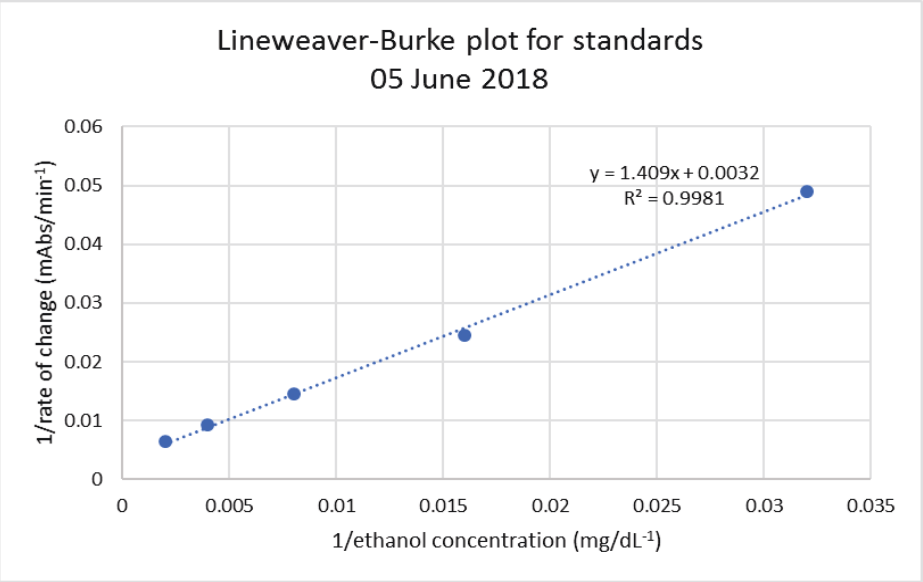

Figure 3*: Representative example of Lineweaver-Burke plot for ethanol*

Part II: The Effect of Lactate and LDH on an Assay without Ethanol

The "ethanol" results generated in the alcohol assay for varying combinations of lactate

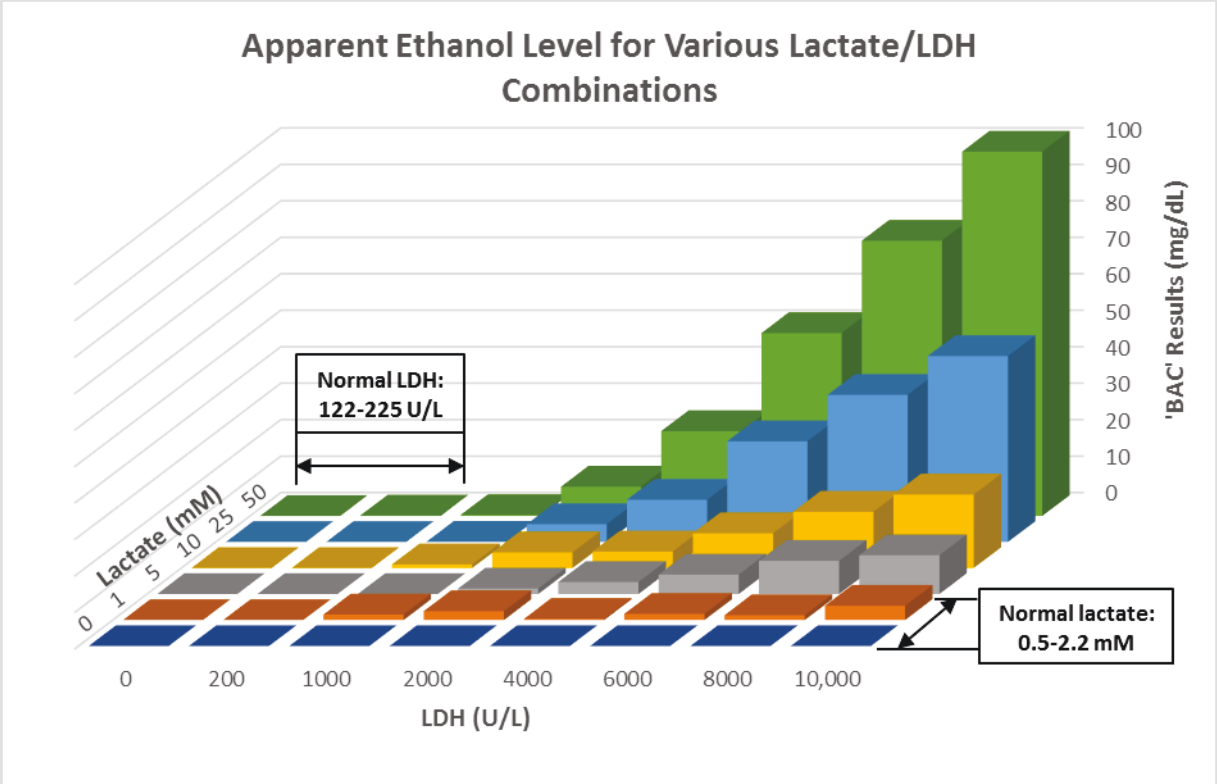

Figure 4: *Apparent ethanol levels* observed in an enzymatic assay containing only ADH, LDH, lactate and NAD⁺ (*Note: The control sample of zero lactate was used as the 'zero point' for its respective matrix of data)*

and LDH, in the absence of ethanol in the reaction mix, are given in Figure 4. As hypothesized, no false positive results were observed in either type of control sample containing no lactate or no LDH. The ethanol results are correlated positively with elevated lactate and LDH concentrations, with values greater than 0.02 g/dL occurring at and above 4000 U/L LDH and 5 mM lactate. No significant false positives (>0.02 g/dL) were observed at physiologically normal levels of lactate and LDH (indicated on Figure 4). At the highest levels of LDH and lactate (10,000 U/L and 50 mM, respectively) the apparent ethanol result reached 0.099 g/dL.

The apparent blood alcohol content observed in false positives for this enzyme assay can be expressed in mg/dL by the equation below (Equation 1) as a function of lactate (mM) and LDH (kU/L). This equation was derived by using the observed linear relationship in the

$$EtOH = ((-0.0018[LDH]^3 + 0.0377[LDH]^2 - 0.0009[LDH]) * [lactate]) - 0.733$$

Equation 1: *Predictive equation for approximate false positive result with known blood levels of lactate and LDH*

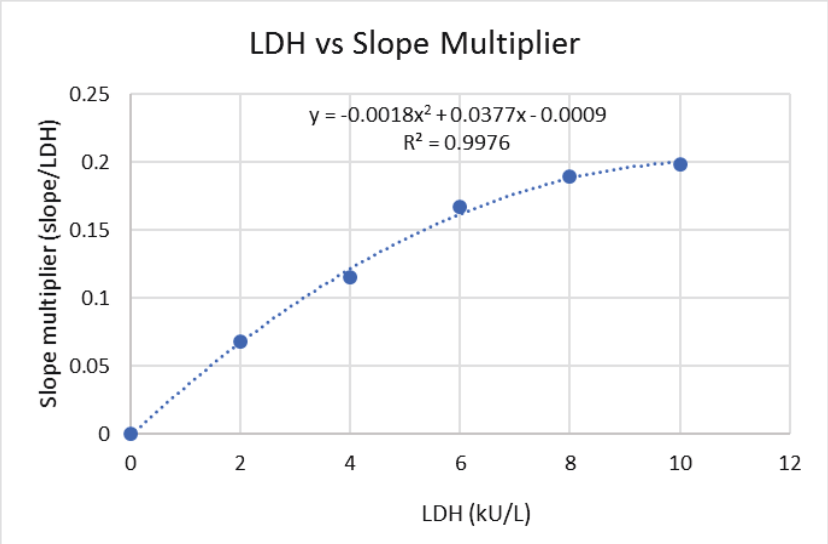

Figure 5: *Slope as a function of LDH concentration for each matrix of data*

perceived ethanol results for each level of LDH as a function of lactate. The slope of this line is dependent on the concentration of LDH; the slope and the LDH concentration have an exponential relationship (see Figure 5). The linear relationship between the apparent ethanol results and the exponential relationship between the slope/LDH concentration were combined

to derive Equation 1. Its validity was tested by inputting the experimental values of lactate and LDH tested in this project, then comparing the results of the equation with the experimental results (see Figure 6). The predicted values correlated reasonably well with the experiment values, especially at higher levels of lactate (see Table 7). This equation may be used to predict approximate false positive magnitudes for values of lactate and LDH concentrations other than those tested in this experiment. However, because this equation was derived based on data from the enzyme assay used at UNH, its numerical results are only applicable to samples analyzed using the same enzyme assay. A similar conceptual approach could be used with another assay to derive an equation for another system.

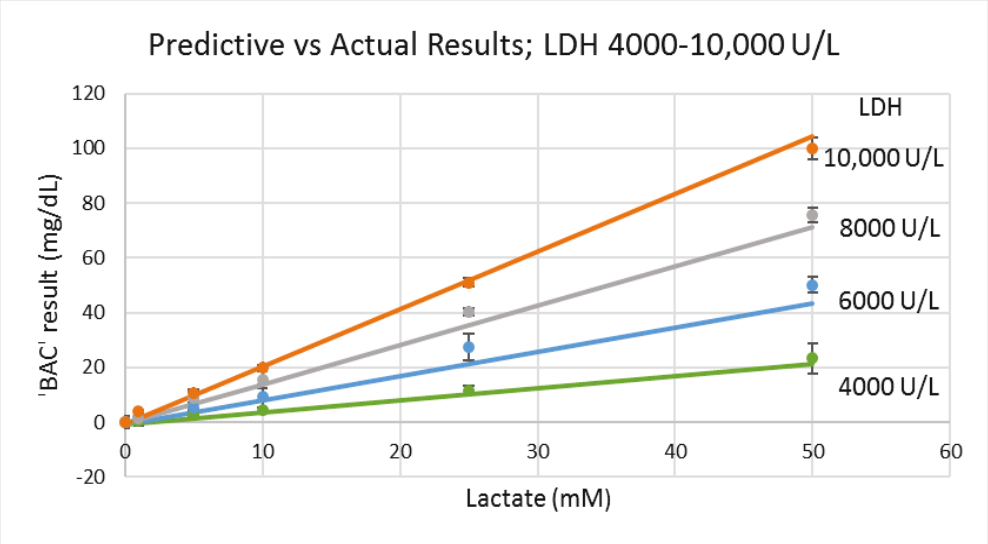

Figure 6: *Accuracy of predictive equation results (solid lines) compared to actual experimental results (data points). Data points include error bars of 2 standard deviations.*

TABLE 7: *Predictive equation results compared with experimental results for a single data matrix [LDH = 8000 U/L]*

Lactate (mM)	Predictive Equation Result (mg/dL)	Predictive Equation Result ±20% Error	Experimental Result (mg/dL)	Percent difference
0	-0.7	(-.88 – -.59)	~0.00	n/a
1	0.7	(.56 – .85)	1.4	48%
5	6.5	(5.2 – 7.7)	9.1	29%
10	13.6	(10.9 – 16.4)	15.4	11%
25	35.2	(28.2 – 42.3)	40.3	13%
50	71.2	(56.9 – 85.4)	75.5	6%

Part III: The Effect of Lactate and LDH on an Assay with Ethanol Standard Solutions

Perceived ethanol results for systems containing 31.25 mg/dL, 62.5 mg/dL, and 125 mg/dL ethanol are represented in Figures 7-9, respectively. The results indicate that the two

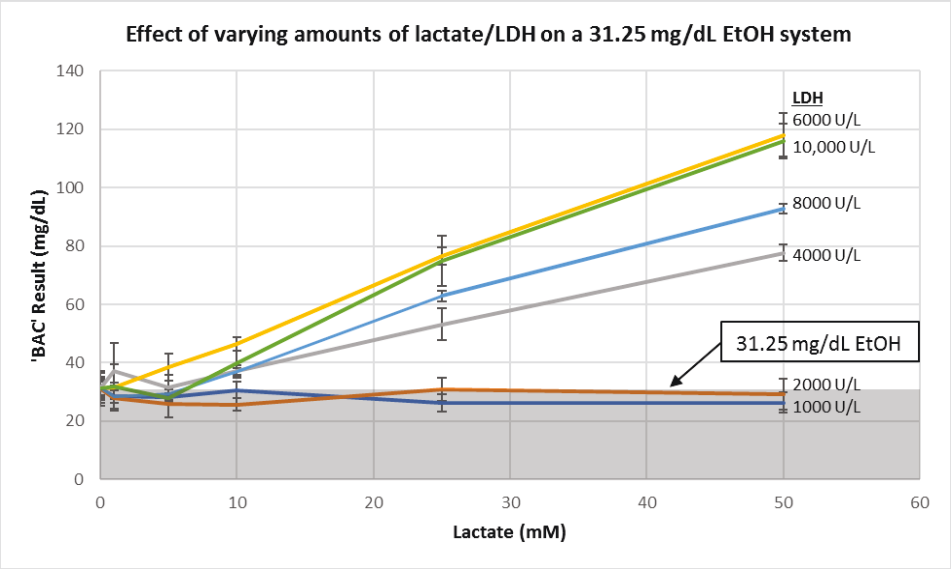

Figure 7: *Apparent ethanol levels observed in an enzymatic assay containing ADH, LDH, lactate, NAD$^+$, and 31.25 mg/dL ethanol. (Error bars = 2 standard deviations)*

enzyme systems (lactate and ethanol) can produce NADH simultaneously. This dual, more rapid production of NADH leads to a higher apparent ethanol result than expected for a certain level of ethanol alone in the system. In each figure, the gray shaded area represents the expected ethanol result for the amount of ethanol actually present in the system. Elevated

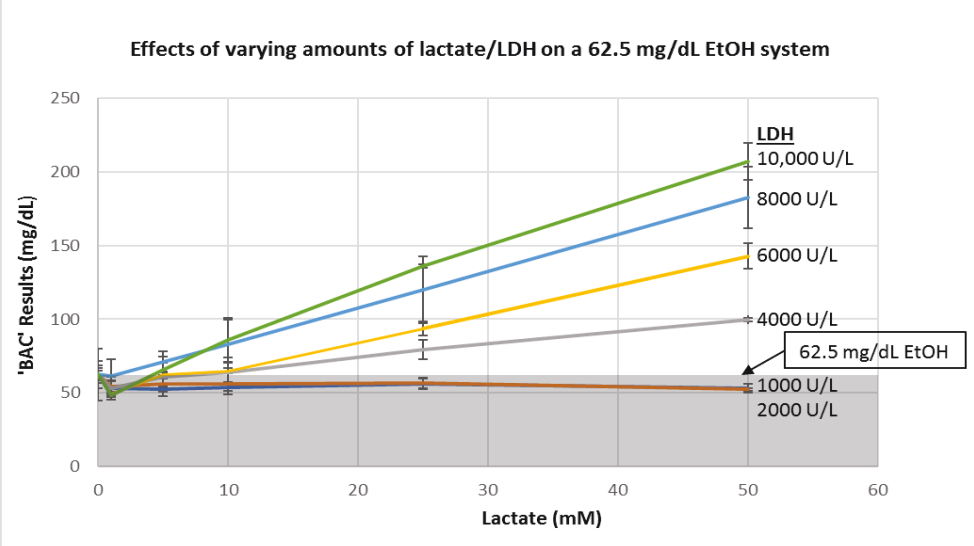

Figure 8: *Apparent ethanol levels observed in an enzymatic assay containing ADH, LDH, lactate, NAD$^+$, and 62.5 mg/dL ethanol. (Error bars = 2 standard deviations)*

perceived ethanol results were observed in all three systems at and above 4000 U/L LDH. As in a system with no ethanol, higher levels of lactate and LDH generally led to higher 'ethanol' results. However, at a low level of ethanol (31.25 mg/dL; Figure 7) the results for 6000 U/L LDH were higher than those for 8000 and 10,000 U/L LDH. The reason for this anomaly is unknown.

It is also apparent that the system with a high level of ethanol (125 mg/dL; Figure 9) exhibited a proportionately smaller signal increase compared to the other two systems. The results for this system also displayed a less linear response as the concentration of lactate was

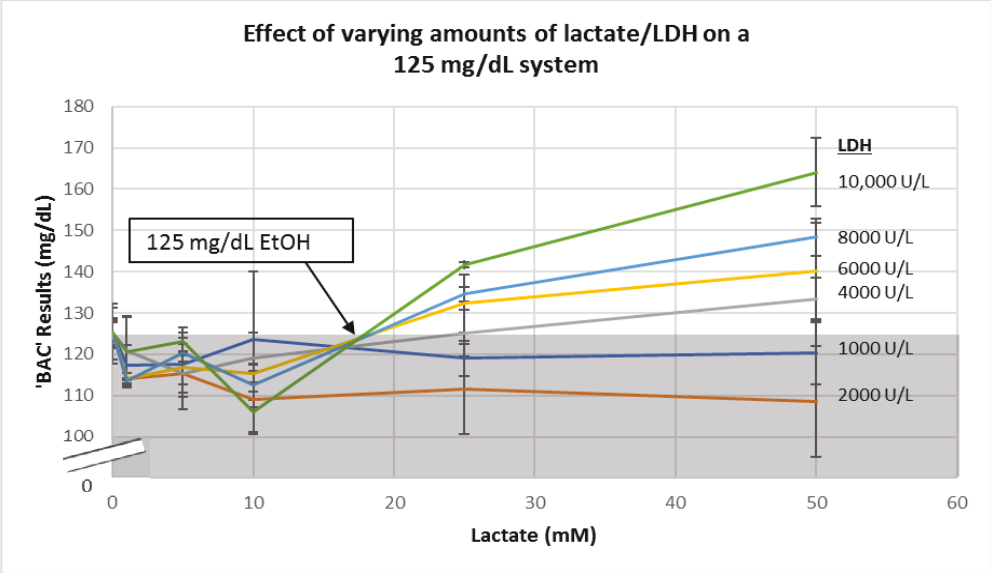

***Figure 9**: Apparent ethanol levels observed in an enzymatic assay containing ADH, LDH, lactate, NAD^+, and 125 mg/dL ethanol. (Error bars = 2 standard deviations)*

increased. These results seem to indicate that some form of interaction occurs between the ethanol reaction and the lactate reaction which is most readily observable at higher levels of ethanol. If the two enzyme reactions occurred with no interaction, it would be reasonable for the magnitude of their combined assay result to be approximately equal to the results of both separate assay results added together. When the results of the separate reactions were compared with the combined reaction, no significant differences were observed for the low and medium levels of ethanol (Figures 10-13). However, at a high level of ethanol (Figures 14-15) the combined result was consistently lower than the sum of the two separate reactions.

This indicates that there may be some kind of interaction or interference between the two reactions when they are occurring simultaneously.

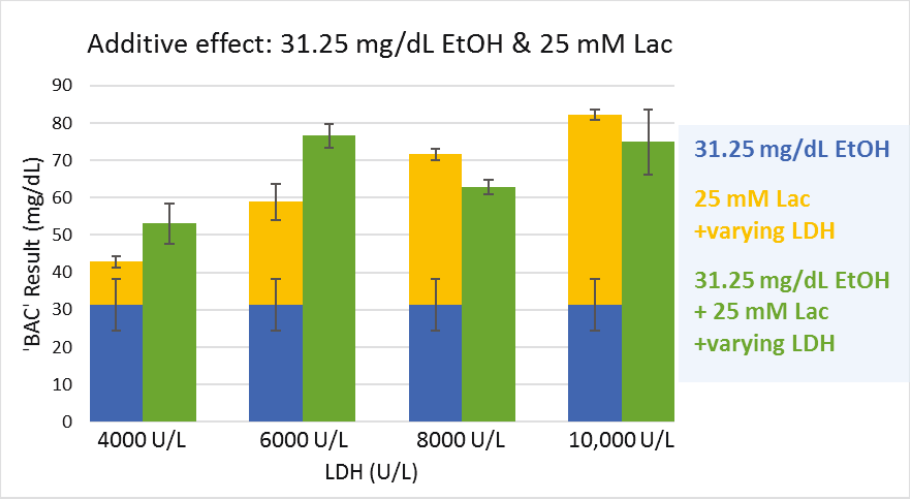

Figure 10: *Results of separate reactions compared with a combined system. No significant differences observed between sum of ethanol/ADH (blue) + lactate/LDH (yellow) and a combined system (green).*

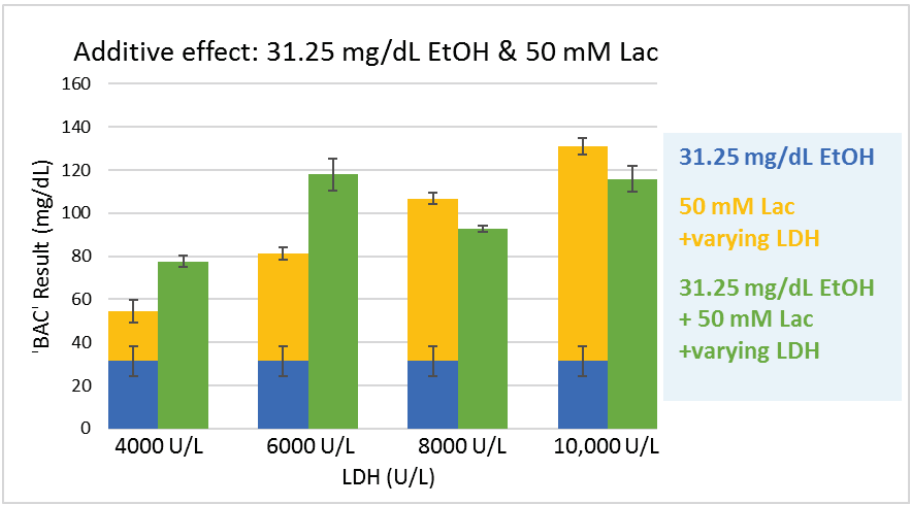

Figure 11: *Results of separate reactions compared with a combined system. No significant differences observed between sum of ethanol/ADH (blue) + lactate/LDH (yellow) and a combined system (green).*

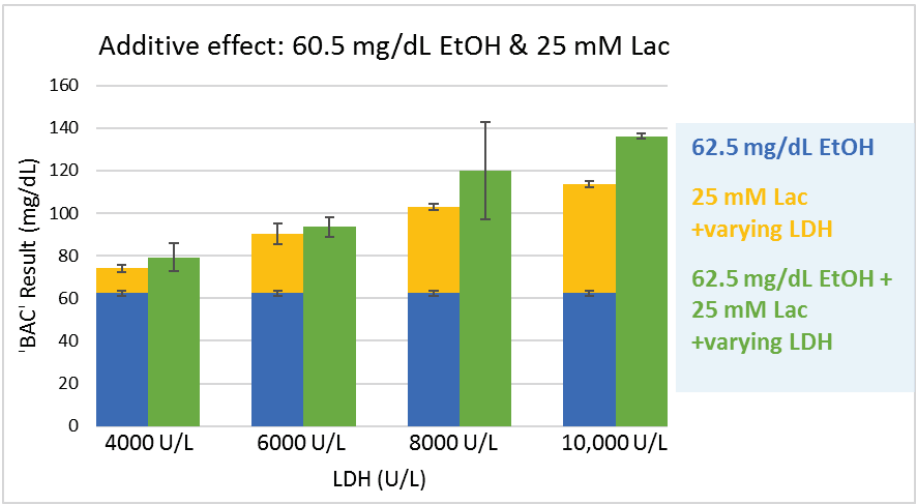

Figure 12: *Results of separate reactions compared with a combined system. No significant differences observed between sum of ethanol/ADH (blue) + lactate/LDH (yellow) and a combined system (green).*

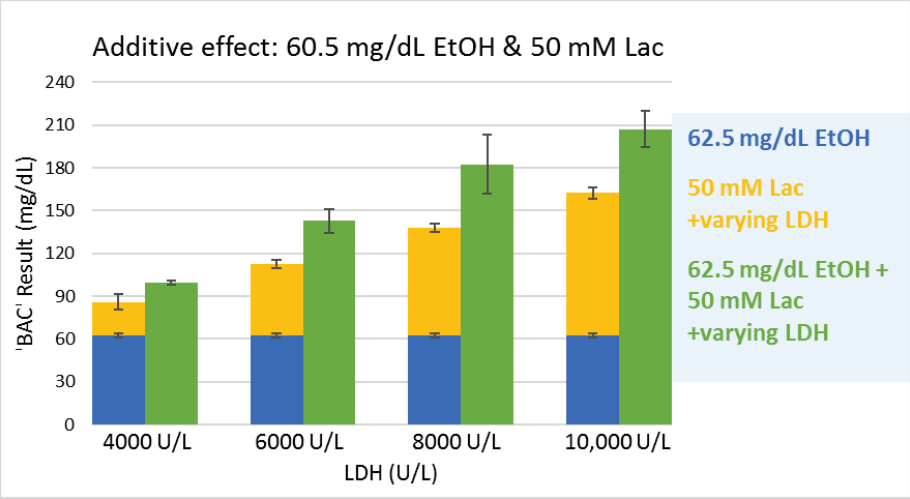

Figure 13: *Results of separate reactions compared with a combined system. No significant differences observed between sum of ethanol/ADH (blue) + lactate/LDH (yellow) and a combined system (green).*

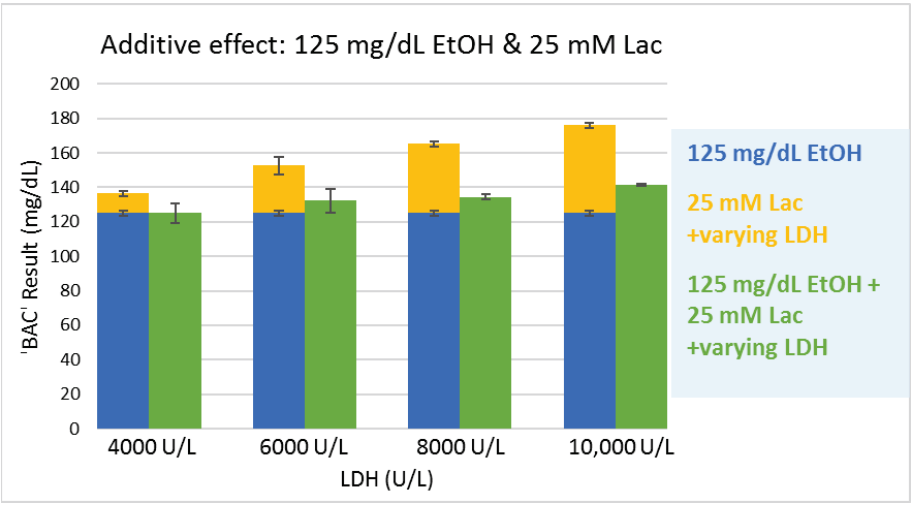

Figure 14*: Results of separate reactions compared with a combined system. Sum of ethanol/ADH (blue) and lactate/LDH (yellow) is consistently higher than the combined system (green).*

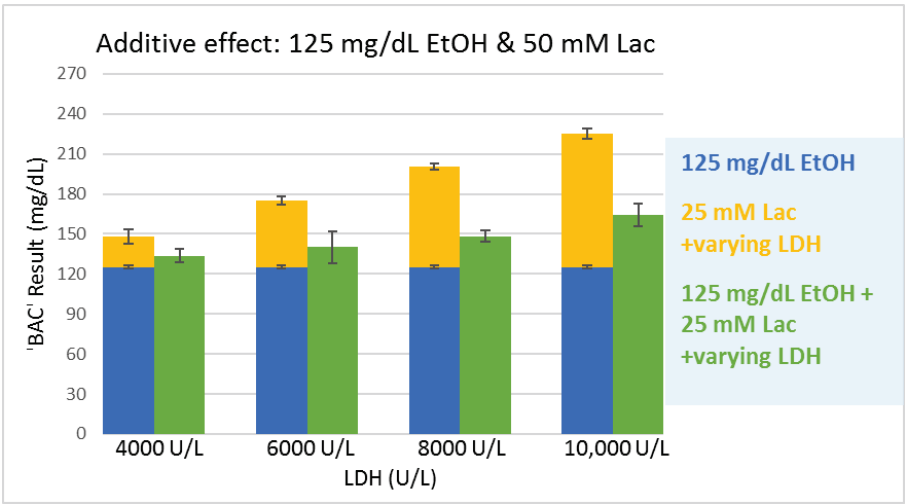

Figure 15: *Results of separate reactions compared with a combined system. Sum of ethanol/ADH (blue) and lactate/LDH (yellow) is consistently higher than the combined system (green).*

To investigate whether ethanol could be directly inhibiting the LDH enzyme, low, medium and high levels of ethanol were added to a lactate/LDH reaction containing no ADH. Therefore, the ethanol would not be metabolized by ADH, so it could not produce NADH via that mechanism. The only signal produced in this version of the assay would be from the lactate/LDH reaction. When these results were compared to lactate/LDH results in the absence of ethanol, no significant differences were observed at LDH 4000 U/L or 6000 U/L (Figures 16-17), even with high levels of ethanol (125 mg/dL). Lower than expected results were observed at LDH 10,000 U/L (Figure 18), which indicates that ethanol is unlikely to be an inhibitor of LDH. If ethanol were inhibiting the LDH enzyme, a reduction of signal would be expected at lower levels of LDH enzyme, not higher levels. Since only the high level of LDH enzyme exhibited a reduction in signal, it is more likely that the ethanol and lactate reactions are competing for the cofactor NAD^+. This hypothesis is supported by the fact that the results are lower than expected when there are high levels of both reactions occurring at once, meaning that there might not be enough cofactor in the system to support both reactions. This is also logical because the assay was optimized using the ethanol reaction by itself, not both reactions simultaneously. This hypothesis could be tested by varying the amount of NAD^+ in the system to determine if the drop in signal still occurred at high levels of both reactions.

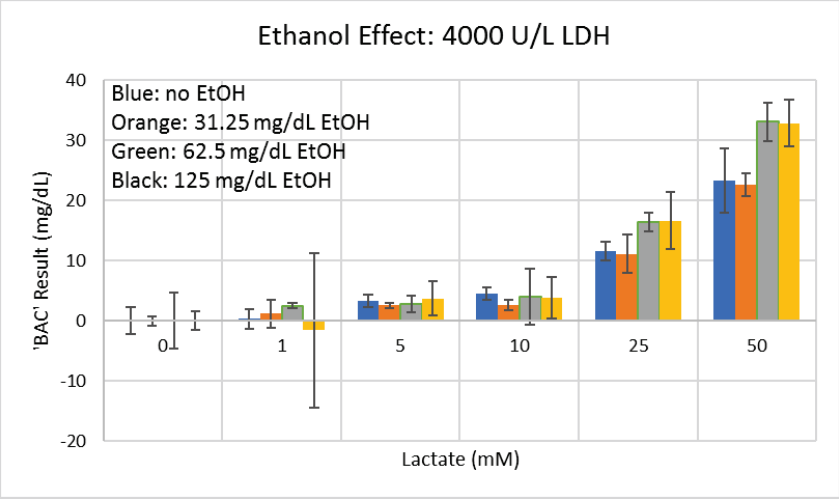

Figure 16: *Effect of ethanol added to lactate/LDH reaction in low, medium and high amounts compared to lactate/LDH with no ethanol present. No significant differences observed.*

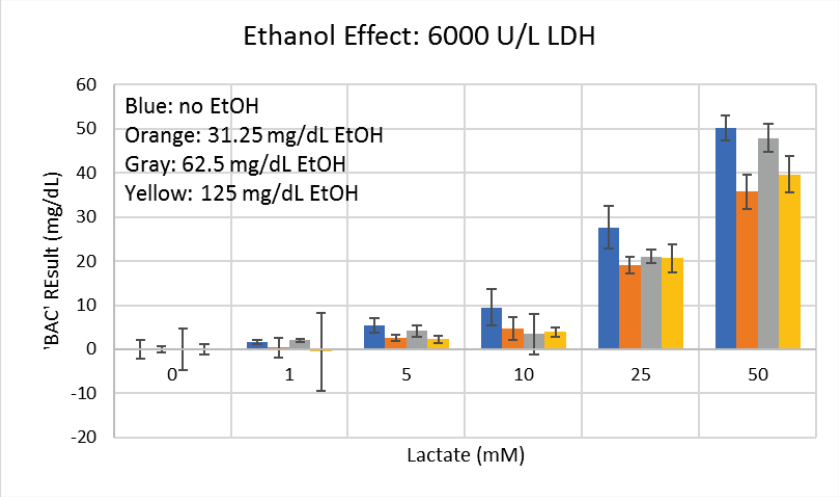

Figure 17*: Effect of ethanol added to lactate/LDH reaction in low, medium and high amounts compared to lactate/LDH with no ethanol present. No significant differences observed.*

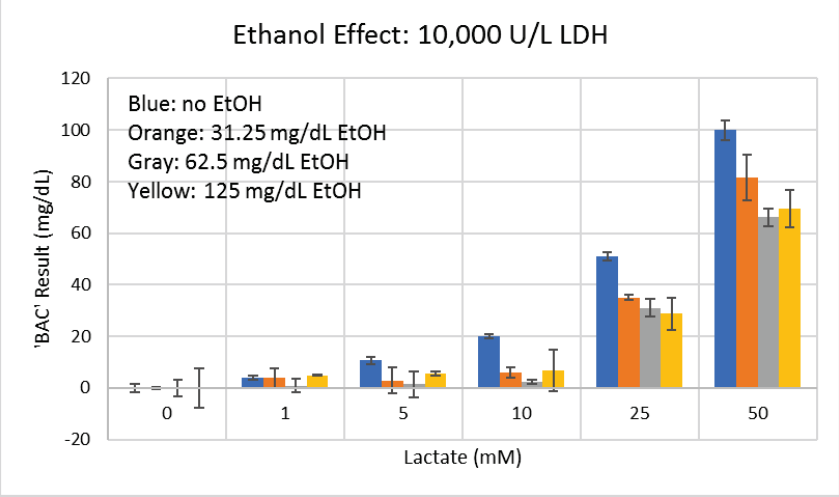

Figure 18: *Effect of ethanol added to lactate/LDH reaction in low, medium and high amounts compared to lactate/LDH with no ethanol present. Lower results observed when ethanol is present.*

Part IV: Verification of Results using a Human Serum Matrix

When samples prepared in human serum were compared to the samples in Part II (a lactate/LDH system with no ethanol), no significant differences were observed. Figure 19 shows a comparison of the results with human serum (blue) and water matrices (yellow). In all but one combination of lactate/LDH, the error bars overlap, indicating that the human serum had similar results to the water matrix within two standard deviations. At the highest combination of lactate (50mM) and LDH (10,000 U/L), the error bars did not overlap, but the percent difference between the results was ~11.76%. These results indicate that the response of a sample in human serum, such as in clinical samples, should have reasonably similar results as the samples tested in Part II of this experiment.

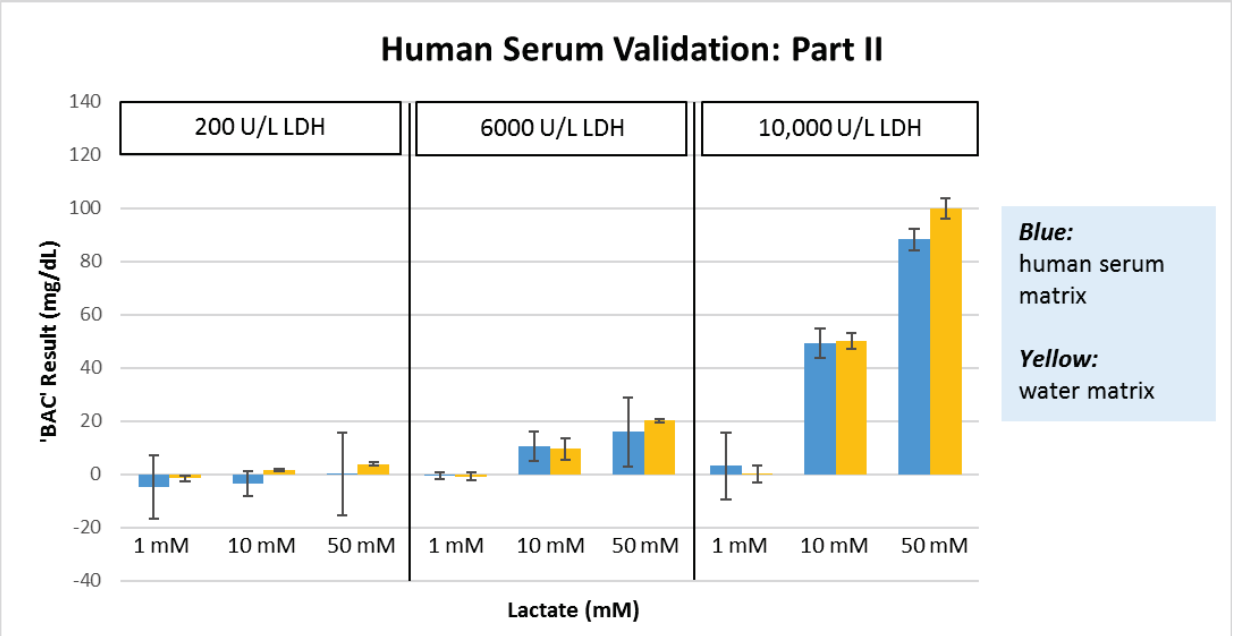

***Figure 19**: Results from samples run in human serum compared to those in water; system containing ADH, NAD^+, LDH, and lactate.*

When samples prepared in human serum were compared to those from Part III, much higher results were observed for the human serum trials (see Table 8). The assay was also run without the cofactor NAD^+ and the ADH enzyme to determine what might be causing the discrepancy. Because there are positive results from the samples with no cofactor added to

the assay, it is likely that the human serum matrix contains some cofactor. This could explain the higher values observed in the human serum as compared to the water matrix. Extra cofactor would not have affected the results in Figure 19 to the same degree, because there was only one enzyme reaction occurring (between lactate and LDH). In the results from Part III, the ethanol and lactate reactions are occurring simultaneously, so there may have been some competition for the cofactor. In this case, extra cofactor in the serum could have increased the rate of reaction by preventing competition for the cofactor.

TABLE 8: *Human serum results compared with water matrix results in a system with 31.25 mg/dL ethanol and 6000 U/L LDH*

Lactate (mM)	Water Matrix Results (mg/dL)	Human Serum Results (mg/dL)	Human Serum [no NAD in assay] (mg/dL)	Human Serum [no ADH in assay] (mg/dL)
1	22.35262	91.82792	18.71938	42.37956
10	38.936	101.2568	25.51718	70.71358
50	115.6277	161.9857	23.66795	137.4986

Quantitative enzyme assays normally have both cofactor and enzyme available in excess so that the rate of reaction is limited only by the amount of substrate (ethanol) introduced in the serum sample. Since this ethanol enzyme assay was optimized and tested with ethanol standards only, there may be plenty of cofactor for a single reaction at a time. But as has been observed in Parts III and IV of this project, an unexpected second reaction could lead to competitive and less predictable results. This could also be a concern with hospital enzyme assays because they are likely developed and verified with ethanol standards as well.

CHAPTER IV: CONCLUSIONS & FUTURE RESEARCH

Conclusions

The results of this project indicate that while interference in the ADH enzymatic assay from a co-reaction between lactate and LDH is rarely observed, but may still be a concern if the interference is unrecognized. The assumption that this interference may have occurred may also undermine the appropriate use or interpretation of valid results of the enzyme assay in either the medical community or legal forums. The data from this project indicates that while false positives are indeed possible (reinforcing previous work in this area), but only when lactate and LDH are both elevated significantly above normal physiological levels. The potential for interference may be recognized by observing the anion gap and the values of ALT/AST; if these are higher than expected, elevated lactate and LDH (respectively) could be contributing the perceived ethanol result.

In a system with no endogenous ethanol, lactate and LDH were found to produce significant false positives, correlating with higher levels of both. The behavior of such a system was documented and an equation was derived to predict approximate false positive magnitudes for combinations of lactate and LDH that were not tested.

In a system with endogenous ethanol, additive effects were observed when both the ethanol and the lactate reactions were occurring simultaneously. This could result in the level of ethanol being overstated based on the assay results. The additive effects were smaller in a system with a high level of endogenous ethanol, so there may be some competition or other interaction between the two reactions that is not fully understood at this time. Ethanol does not appear to be a substrate (BRENDA) or inhibitor for LDH. Competition for the cofactor NAD^+ may be a potential explanation, although further research on this topic is needed.

Future Research

Further experimentation is needed to clarify the additive effects of ethanol, lactate, and LDH in a system with some level of endogenous ethanol. In particular, systems with high levels of endogenous ethanol should be examined because unexpected results were observed in the system with 125 mg/dL ethanol. Systems with 250 mg/dL ethanol or higher might provide more insight into the behavior of a system with both reactions occurring simultaneously, as well as the impact of ethanol on the lactate/LDH reaction. A lactate/LDH assay could also be developed and varying amounts of ethanol could be added to determine its influence on the lactate/LDH reaction.

Another experiment that might clarify the effects of a combination system could be conducted by varying the amount of NAD^+ present to determine if it is a limiting factor. If so, it might be possible to reoptimize the ethanol assay with an excess of NAD^+ so that the reaction speed is completely controlled by the amount of substrate introduced in the sample.

In addition to these in vitro studies, it would also be useful to conduct survey studies to determine some larger questions about the occurrence of this interference in a clinical setting. For example, how often does elevated lactate and LDH occur in trauma victims as well as illness victims? Historically, have ALT/AST hepatic enzyme values been shown to accurately predict the level of LDH present in the blood? It would be useful to understand how prevalent elevated levels of lactate and LDH are clinically, and whether the traditional methods of estimating their concentrations are reliable. These studies, along with the work conducted on this project, will continue to deepen the understanding of these false positives and provide valuable information on how to interpret these results in a legal setting.

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