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Comparison of Qualitative and Quantitative Clinical Assays in Suspected Ethylene Glycol Exposure

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Comparison of Qualitative and Quantitative Clinical Assays in Suspected Ethylene Glycol Exposure

By Michael David Schwartz

Background: Availability of rapid analysis for suspected ethylene glycol (EG) exposure remains an unmet need in medical toxicology practice. Ethylene glycol poisoning is a significant source of morbidity and mortality in the U.S. with over 5000 cases of poisoning reported each year to poison control centers.[1] Mass poisonings from methanol-tainted liquor and ethylene and diethylene glycol-contaminated pharmaceuticals have occurred worldwide. Lack of a clinical assay for EG exposure leads to presumptive therapy with expensive antidotes and consumption of hospital resources until the diagnosis can be confirmed. *Objective*: To evaluate the assay performance (sensitivity, specificity, positive predictive value, negative predictive value, correlation) of a i) veterinary point-of-care (POC) qualitative (colorimetric) EG test kit and an ii) enzymatic, chemistry auto-analyzer-based quantitative EG assay. *Methods*: We conducted a cross sectional study (n=56) of the REACT EGT kit (Allelic Biosystems, Kearneyville, WV) and the ZBx LQ Ethylene Glycol Assay (Clinitox Diagnostics, Toronto, Canada) on cases of suspected EG intoxication referred to our poison center. Assay results were compared to criterion standard testing for EG exposure using gas chromatography/mass spectrometry. *Results*: The colorimetric assay lacked specificity for true EG exposure; multiple interferences preclude its clinical utility. The ZBx LQ Ethylene Glycol assay performed with 100% sensitivity and 100% specificity for clinically significant EG exposure (range of EG serum concentrations 6.3 to 620 mg/dl). Method comparison of the assay with GC/MS demonstrated excellent correlation (r=0.9803; p=0.000). *Discussion*: The ZBx LQ Ethylene Glycol assay's performance, ease of adaptability to a hospital clinical laboratory's chemistry platform, and low cost, make it useful for the rapid laboratory diagnosis of EG exposure. The sample size in our study was small; a larger, pivotal study and eventual regulatory approval are required before the assay becomes widely available. *Conclusion*: The ZBx LQ Ethylene Glycol assay fills a significant gap in the routine availability of reliable EG clinical testing.

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Dr. Bhushan Kapur, University of Toronto, served as an external reviewer of my work, both mentoring and offering guidance with the completion of this thesis; this included a review of my statistical calculations. Any remaining errors are mine alone.

Collaborating healthcare systems and hospitals in the metropolitan Atlanta area assisted with case identification and served as sites of enrollment.

The staff of the Georgia Poison Center and its medical director Dr. Robert Geller have always served the public health of Georgia's citizens and stand ever ready to provide care to poisoned patients in this state. Their participation and assistance with this study is a testament to that.

A special thanks to my wife Catherine, who stood by me as I spent triple the amount of time usually required to earn this, my very, very last degree…

Finally, no research in human medicine is possible without the willingness of patients to give of themselves to help us learn so that we may better help others. Without their altruism, this study would not have been possible.

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INTRODUCTION AND BACKGROUND

Ethylene glycol (EG; CAS No. 107-21-1) is the toxic chemical found in automotive antifreeze. EG poisoning is fairly common in the United States, carries significant morbidity and mortality, and results in significant healthcare expenditures during treatment. In 2009, 5282 cases of EG exposure were reported to the American Association of Poison Control Centers (AAPCC),[1] of which 3230 were in adults. In total, 30 deaths occurred and 134 cases with major morbidity were reported. However, the number of patients in which the diagnosis was suspected, and who underwent expensive antidotal therapy and/or invasive hemodialysis – before the diagnosis was ruled-out – was likely many times the number reported to the AAPCC.

EG poisoning occurs as a result of ingestion of EG-containing automotive antifreeze and other commercial products. EG itself is intoxicating, and some self-poisonings result from the intentional ingestion of EG as a substitute for alcohol. EG is metabolized via the *alcohol dehydrogenase* (ADH) system to glycolic acid, and further metabolized to oxalate by *aldehyde dehydrogenase*. The glycolic acid intermediate causes the characteristic high-anion gap metabolic acidosis associated with EG ingestion. Oxalate accumulates in the renal tubules and accounts for the end organ toxicity and significant morbidity and mortality. EG has a volume of distribution in the body (Vd) of 0.6 L/kg and an elimination half-life of 3-8.6 hours with normal renal function. This half-life is prolonged when antidotal inhibition of ADH – with ethanol (17-18 hours) or fomepizole (14-17 hours) – is instituted. EG ingestion presents with similar signs and symptoms as alcohol intoxication: altered mental status (AMS) and signs and symptoms of inebriation. A history of reported or suspected EG ingestion may be given. Diagnostic testing for EG is currently not available in most hospital clinical laboratories. Serum must be sent to reference laboratories to confirm the presence or absence of EG a process takes hours to days. Medical management decisions must be made based on clinical suspicion and instituted before the diagnosis is confirmed or ruled out. This may result in some patients undergoing

unnecessary, expensive, and potentially harmful therapies. If the diagnosis is not made, and therapy delayed, renal failure – often necessitating emergent dialysis - may occur.

EG quantitative levels are obtained by Gas Chromatography/Mass Spectrometry (GC/MS) in a reference laboratory. This assay is not available in most hospitals' clinical laboratories, and the confirmatory result may not be available for more than 24 hours (owing to sample transport and analysis times), thus delaying confirmation of the diagnosis. Even in very large tertiary care hospitals, which might have a GC/MS instrument, results may not be available in a timely fashion. For example, metropolitan Atlanta hospitals require transport of samples to a reference lab with results available to poison center toxicologists and treating clinicians after a mean delay of 12 hours after Emergency Department (ED) presentation. This is considered optimal management at our institutions in Atlanta; the process at smaller, community, or rural Georgia hospitals, is much more problematic, and the times involved are much longer.

Because the risk of significant morbidity and mortality is high, nearly all suspected cases are treated with one of three modalities (ethanol, fomepizole, hemodialysis) before the diagnosis of ethylene glycol poisoning can be confirmed by the reference laboratory. The number of patients treated is much higher than the number actually poisoned EG – a clear consequence of the lack of a suitably robust screening test. Antidotal treatment in suspected exposure included, historically, enzymatic block of *alcohol dehydrogenase* with oral or parenteral ethanol administration. Currently, this metabolic block can be accomplished with a safer, more easily administered antidote, fomepizol (Antizol™, Paladin Labs) which has a retail cost of $1900 a dose. Fomepizole has over 8000 times the binding affinity for *alcohol dehydrogenase* than does EG, and effectively blocks any metabolism of EG to glycolic acid or oxalate until the non-toxic, un-metabolized EG can be removed by hemodialysis or urinary excretion.[2]

Often multiple doses are administered while laboratory confirmation is performed. Antidotal therapy and dialysis are expensive and carry inherent risks. Additionally, nephrologists are often reluctant to perform dialysis in patients without clear evidence of EG poisoning such as laboratory confirmation with GC/MS. Thus, suspected EG intoxication has been the subject of considerable effort to develop a more rapid clinical assay to confirm or rule-out the diagnosis in a clinically relevant time frame to inform physician decision-making.

Veterinary Qualitative POC Assays

Allelic Biosystems (Kearneysville, WV) markets a qualitative veterinary assay (REACT EGT kit) used *in vivo* for the rapid detection of EG poisoning in canine and feline species.[3] This assay is an inexpensive, liquid reagent based, cage-side assay with a reported limit of detection of EG of 50 mg/dl. The assay involves a simple oxidation and colorimetric reaction and can be completed in 15 minutes. Blood components are precipitated with sodium tungstate. EG in serum is oxidized with periodate to formaldehyde. Formaldehyde forms a colored compound with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole at alkaline pH. Investigators in New York have used the kit on human serum samples from known and suspected EG exposures (n=24; 15 positive for EG; range of EG 27-281 mg/dl) and demonstrated a Sensitivity of 100% and specificity of 88.8%.[4]

Kacey Inc. (Asheville, NC) markets the Kacey Ethylene Glycol Test. The kit uses a colorimetric test strip which forms a blue signal if EG is present when a 20 ul aliquot of plasma is applied to the indicator. The intensity of the color change is reported to be proportional to the concentration of EG present and a reference color chart is provided on the test strip bottle.[5] The manufacturer tested 50 spiked samples with 0, 20, 50, and 100 mg/dl of using the Kacey kit. Results were reported as follows: 0 mg/dl (50/50 correctly identified), 20 mg/dl (49/50), 50 mg/dl (48/50), 100 mg/dl (50/50). In addition, the Kacey test has been used to analyze 57 feline

plasma samples spiked with 20, 60, or 80 mg/dl of EG.[6] There was substantial agreement between two reviewers (κ=0.7) at the two lower concentrations, and moderate agreement between the two reviewers (κ=0.5) at the highest concentration. Sensitivity ranged from a low of 56% at 20 mg/dl to a high of 100% at 80 mg/dl, while specificity ranged from 25% (80 mg/dl) to 77% (20 mg/dl).[6] The authors point out that the LD_{50} for EG concentration in cats is 20 mg/dl.

Veterinary Quantitative Enzymatic Assay

Catechem inc. (Oxford, CT) has developed a veterinary assay (Catechem ethylene glycol test) which utilizes bacterial source *glycerol dehydrogenase* to oxidize EG. NAD is converted to NADH which is detected by spectrophotometry (340 nm) and the absorption is proportional to the concentration of EG. Scherk, et al, spiked serum and plasma samples obtained from canine (n=6) and feline (n=4) patients with 0, 20, 60, or 100 mg/dl EG and obtained coefficient of variation (2.1%), average bias of all Catechem values obtained compared to GC/MS (+7.83 mg/dl; 95% CI -2.1 to 17.8 mg/dl), and correlation with GC/MS of 0.9939.[7] Mean EG concentrations in un-spiked serum and plasma samples (0 mg/dl EG) ranged from 0.2 to 2.4 mg/dl. Recognizing the potential interference in the *glycerol dehydrogenase* method from exogenously administered medications containing propylene glycol (PG), the investigators spiked plasma samples with diazepam (2.5, 5, 25 mcg/ml) which contains PG as a diluent; the highest EG concentration obtained was 10 mg/dl in the high-diazepam concentration samples.[7]

Juenke, et al, compared the precision and correlation of the Catechem assay in prepared plasma and patient samples at 5 levels of EG concentration (5, 10, 50, 100, 200 mg/dl; numer of samples not reported).[8] The CV varied from 2.1% at 50 mg/dl level to 7.7% at the 100 and 200 mg/dl levels. The correlation with GC/MS was r=0.99 and the limit of detection (LOD) was 5 mg/dl. PG reacted at greater than 100% cross reactivity, however GC negative samples (n=30) in which no other glycol was present, tested negative with the Catechem assay.

Human Quantitative Enzymatic Assay

Clinitox Diagnostix (Toronto, Ontario, Canada) has completed development and performance testing of an enzymatic assay for the quantitative determination of ethylene glycol in human serum on most automated clinical chemistry analyzers. The assay is already approved and in Clinical Use in Canada and Europe.

The ZBx LQ Ethylene Glycol Assay is based on the affinity of the enzyme *glycerol dehydrogenase* (GDH, EC 1.1.1.6) from the bacteria to catalyze the oxidation-reduction reaction of EG in the presence of nicotinamide adenine dinucleotide (NAD).[9] The method monitors the absorbance at 340 nm and the increase in absorbance is directly proportional to the concentration of EG in the serum sample. The following enzymatic reaction underlies the assay:

$$\text{Ethylene glycol} + \text{NAD} \xrightarrow{GDH} \text{NADH} + \text{Glycoaldehyde} + H^+$$

Performance testing of the ZBx LQ Ethylene Glycol Assay by the manufacturer utilized 73 patient serum samples (33 positive for EG) and found a correlation of 0.9725 compared with gas chromatography/flame ionization detection (GC/FID).[9] The LOD was reported to be 5 mg/dl and the limit of quantitation (LOQ) was 12 mg/dl; the method was linear over the range of EG concentrations from 0 to 300 mg/dl. High concentrations of PG (>152 mg/dl) and 2,3-butanediol (>11 mg/dl) resulted in falsely elevated EG readings; numerous additional toxic alcohols, ethanol, glycols, and volatile compounds did not result in false positive EG readings.

METHODS

Study Sample

This study was approved by the Institutional Review Board (IRB) of Emory University. The study was conducted at 5 metropolitan Atlanta healthcare systems comprising 16 hospitals. All participating hospital systems either independently provided their own IRB approval (1) or deferred to Emory University's IRB approval and served as sites of enrollment (4) after providing the investigators with permission letters. Patients eligible for enrollment in the study were identified when their history or clinical presentation were suggestive for EG exposure. Cases were primarily identified from calls to the Georgia Poison Center (GPC) from participating hospitals requesting guidance on diagnosis and treatment of suspected EG exposure. All enrolled cases or their responsible party signed both Informed Consent and HIPPA waiver before acceptance into the study. Enrolled patient's agreed to allow investigators to utilize a previously collected serum sample for the study; no additional phlebotomy was performed for study purposes only. The study was conducted independently of clinical management and study results were not used to guide diagnosis or care of enrolled patients, most of whom were managed clinically by medical toxicologists who were also study investigators. Accrual occurred between August 1, 2010 and July 31, 2012.

Enrolled patients were assigned a study number and relevant demographic (age, gender) and biochemical parameters were abstracted onto a standard study data collection tool. When available, exposure parameters (EG amount; time of ingestion) were abstracted. Serum samples were transported in refrigerated shipping containers to the study site where they were maintained at 4C until analysis by either of the two experimental assays. Data were entered on a password-protected, Excel (Microsoft, Redmond, WA) spreadsheet until data analysis. The result of reference laboratory testing was entered by subject study number and all identifiers were removed.

Veterinary REACT EGT Kit

Analysis using the REACT EGT kit involved two investigators who interpreted the presence or absence of a color change; a kappa statistic was calculated for inter-rater reliability. Investigators were not blinded to case status. EGT kits were maintained refrigerated until use as recommended by the manufacturer. The REACT EGT kit uses periodate to reduce EG to formaldehyde which was detected using a colorimetric indicator. Briefly, the vial of buffer solution is pipetted into the indicator powder to dissolve. The resulting solution is transferred to a third vial containing the activating powder. The resultant mixture is evenly divided between two vials, one to serve as a negative control and one for the assay. Forty microliters of serum is pipetted into the final mixture and any color change was read after 1 minute.

ZBx LQ Ethylene Glycol Assay

The ZBx LQ Ethylene Glycol Assay requires two liquid reagents (ZBx LQ Ethylene Glycol Sample Diluent; ZBx LQ Ethylene Glycol Activator Reagent) which are stable for 60 days at 2-8^{o}C once opened. The assay includes two Control solutions (EG 56 mg/dl, range 46-66 mg/dl and 248 mg/dl, range 208-288 mg/dl) to monitor the quality and performance of the assay on a daily basis. Presentation of the assay is in a 40 test or 80 test package. Analyses with the ZBx LQ Ethylene Glycol Assay for our study was kindly performed by Clinitox Diagnostix using a Olympus AU600 Chemistry analyzer (Olympus Systems, Center Valley, PA). A 500 ul aliquot of serum from a subset of enrolled patients (n=35) were maintained frozen at -4C and shipped on dry ice to Clinitox Diagnostix. All specimens were identified only by study number and collaborators at Clinitox Diagnostix were blinded to patient status.

Study Confirmatory EG Testing and Testing for Interfering Substances

An aliquot of each enrolled patient's serum was sent for confirmatory EG analysis using GC/MS (NMS Labs, Willow Grove, PA). Enrolled cases had clinical EG analyses performed using GC/MS for diagnostic purposes (Quest Diagnostics, Tucker, GA). However, since the specimen for expedited clinical EG testing may have been drawn at a different time than the serum specimen provided for the study only the EG result from NMS Labs was used by study investigators as definitive evidence of exposure status. REACT EGT kit analysis, ZBx LQ Ethylene Glycol assay, and reference lab testing for EG were all performed on aliquots of the same serum specimen.

In addition to EG, GC/MS analysis for propylene glycol (PG), diethylene glycol (DEG), and volatile organic compounds were performed. PG is a common pharmaceutical additive and diluent suspected to interfere with the performance of the REACT EGT kit. PG is also a component of putative "non-toxic" newer antifreeze preparations and may have been ingested *in lieu of* EG unknowingly by patients who offered only a history of "antifreeze" ingestion. Volatile compounds such as toxic alcohols, ethanol, solvents, and aldehydes are frequent co-ingestants in suspected EG exposure cases. Some of these volatile compounds are known or hypothesized to share the *alcohol dehydrogenase/aldehyde dehydrogenase* metabolic pathway with ethylene glycol. Their presence could affect the quantity of EG available for conversion by *glycerol dehydrogenase*/NAD by inhibiting or inducing the rate of EG metabolism by *alcohol dehydrogenase*. Investigators performed analysis for volatiles to validate the lack of interference of these additional analytes – potentially present in a variety of automotive fluid products – which could lead to unexpected differences between the ZBx LQ Ethylene Glycol assay result and the GC/MS method.

Data Analysis

Descriptive summary statistics and frequency and percentage distributions were used to analyze the study sample. The inter-rater reliability between results obtained with the REACT EGT kit was assessed through the kappa measure of agreement. Sensitivity and specificity analyses were performed to compare the accuracy of the REACT EGT kit and ZBx LQ Ethylene Glycol Assay with true exposure status as determined by reference laboratory EG test results. Correlation between quantitative results obtained with the ZBx LQ Ethylene Glycol Assay and the EG result obtained by GC/MS was calculated using the Pearson's correlation (r).

RESULTS

Study Sample

Seventy-two patients were eligible for the study; twelve refused participation and another 4 were not included in the data analysis (Figure 1). The final study cohort was 56 (22 positive for EG exposure; 34 negative). Forty-one patient samples were tested using the REACT EGT kit (15 positive, 26 negative). Thirty-five patient samples were analyzed using the ZBx LQ Ethylene Glycol assay (11 positive, 24 negative). Eighteen patient samples were used on both assays.

Table 1 provides summary statistics for the study sample stratified by EG exposure status. Summary statistics in Table 1 show that the average age of the patients negative for EG was slightly higher (43.85 years) than the average age of the patients tested positive (43.14 years). The mean creatinine concentration was lower (1.64 mg/dl) for patients positive for EG than that of patients negative for EG (2.38 mg/dl). The mean bicarbonate concentration was also found to be higher among patients negative for EG (20.09 mEq/L) than for positive patients (15.95 mEq/L). Among patients who co-ingested ethanol, the concentration of EtOH was higher (40.83 mg/dl) amongst those who were EG negative. Not surprisingly, the serum Osm, Osm gap, and AG were higher among patients positive for EG.

Summary statistics of the study sample stratified over the type (enrolled, refused) were also computed and are presented in Table 2 to investigate potential systematic bias resulting from study inclusion/non-participation. The mean levels of clinical ethanol, serum Osm and AG were found to be higher for patients who refused than patients who enrolled. Table 2 shows that among the enrolled patients, 21.3% co-ingested alcohol, while 62.5% of patients who refused enrollment had elevated ethanol concentration. Alcohol intoxication may have played a role in patient's refusal to participate in a study. In addition, EG may sometimes be consumed as a substitute intoxicant when ethanol is not available. The higher frequency of ethanol intoxication

among non-participants may have biased the study by selecting out participants at a lower risk (based on access to, and consumption of, ethanol) for EG exposure.

Sensitivity and Specificity of the REACT EGT Kit

Table 3 presents the cross tabulation of the test results from REACT EGT kit and NMS EG. Patients tested positive by determination of Observer 1 or Observer 2 were considered positive on the kit and compared with the test results from the NMS EG to determine sensitivity and specificity. Results in Table 3 show that the REACT EGT kit had a sensitivity value 1.00 and a specificity value 0.04. The sensitivity measure of 1.00 implies that all the patients tested positive on NMS EG were also tested positive on the REACT kit with no false negatives. However, the specificity measure of 0.04 implies that among patients negative for EG on reference laboratory testing, only 4% were also negative on the REACT EGT kit, suggesting a 96% false positive rate. Prevalence of confirmed EG exposure among patients presenting with suspected EG poisoning (based on a history of EG ingestion or a metabolic acidemia, pH <7.30 or serum bicarbonate <18 mEq/L) in our state was determined by Sutter, et al, during a 15 month period in 2007-8 (prev=0.44; n=102).[10] The positive predictive value (PPV) of the REACT EGT Kit is 0.45 and the negative predictive value (NPV) is 1.00 suggesting that 55% of positive findings using the EGT Kit in our patient population will be false positives. As a test for confirming EG exposure and thus making a clinical decision about the administration of costly antidotal therapy, dialysis, and instituting intensive medical care, the high false positive rate of the EGT Kit suggests a large number of suspected EG intoxications will be treated unnecessarily.

The results of the inter-rater analysis are $\kappa=0.38$ with $p < 0.05$. This measure of agreement, while statistically significant, was only marginally convincing. As a general rule, values of kappa from 0.21 to 0.40 are considered fair, 0.40 to 0.59 are moderate, 0.60 to 0.79 substantial, and 0.80 outstanding.[11] The study data showed a fair agreement between the diagnoses between

raters evaluating a color change for the REACT EGT kit. One explanation for this low kappa statistic might be the virtual absence of kits for which no color change was observed.

Comparison of ZBx LQ Ethylene Glycol Assay versus EG exposure status

Results from ZBx LQ Ethylene Glycol Assay and NMS EG concentration were also compared using sensitivity and specificity analyses. The results are presented in Table 5. It was observed that both the sensitivity and specificity values for the ZBx LQ Ethylene Glycol Assay when compared with EG exposure status were found to be 1.00 implying no false negative and no false positive findings. Based on a prevalence of confirmed EG exposure of 0.44 (Sutter, et al) the PPV is 1.00 and the NPV is 1.00. The high PPV suggests that no positive test results using the ZBx LQ EG Assay are false positive, assuring that resource intensive interventions (antidote, dialysis, intensive care) are being performed on patients with true EG poisoning. Conversely, the high NPV suggests that patients with a negative test result can avoid unnecessary therapy for suspected EG poisoning (no false negatives). Measures of quantitative results from the ZBx LQ Ethylene Glycol Assay and the EG concentration obtained by GC/MS for the exposed patients were highly correlated (r=0.9803; p=0.000) (Figure 2).

Sensitivity and Specificity of the REACT EGT kit versus EG exposure status after stratifying over propylene glycol status

Finally, sensitivity and specificity analyses were performed for the REACT EGT kit versus EG status after stratifying for the propylene glycol status (negative, positive) to examine whether propylene glycol is an interfering substance that inappropriately renders the EGT kit results positive. The analyses results are showed in Table 6.

Results in Table 6 show that irrespective of the result of NMS PG (negative or positive), the sensitivity value of Vet Kit tool is 1.00 implying no false negatives in any of the cases. However,

the specificity value is reduced from 0.10 (when NMS PG result was negative) to 0.00 (when NMS PG result was positive). A reduction in specificity implies an increase in the false positive rate in the presence of PG, further evidence that PG is just one interfering substance inappropriately triggering a color change for this assay.

Additional Toxicological Findings

The most frequent finding on reference testing was PG (n=31). PG concentrations were in the range 1-38 mg/dl. The finding of a detectable PG level did not correlate well with EG exposure status, thus, while some PG may be present in current antifreeze formulations, the likely explanation for the finding is PG which is present as a pharmaceutical additive in oral or parenteral medications taken or administered by the patients. Some specimen transport tubes are made of polypropylene: in order to be sure that PG was not present in specimen tubes used for patient samples in this study, empty blood collection and serum transport tubes were sent to NMS Labs and analyzed for PG. All tests were run in duplicate and all collection and transport tubes were negative for PG.

Acetone was the second most frequent finding in volatile compound analysis (n=14); in cases of confirmed isopropanol ingestion (n=3) the acetone concentration was markedly elevated as would be expected. Two cases of methanol co-ingestion were identified (1 known from history; 1 unsuspected); one of which occurred in an EG positive patient. A surreptitious toluene exposure (5.08 mcg/ml) was identified by volatiles analysis in a patient positive for EG. One clinically severe exposure to xylenes by history was confirmed (p-xylene 5.2 mcg/ml; m-xylene 8.3 mcg/ml; o-xylene 2.0 mcg/ml) on volatiles analysis.

DISCUSSION

REACT EGT Kit Performance

The initial study exploring a clinically useful POC test for EG exposure was conducted by Long, et al, at the NYC Poison Center.[4] The authors retrospectively analyzed a convenience sample of (n=24) specimens from confirmed positive and negative exposures using the EGTK, an earlier version of the REACT EGT veterinary kit. The authors found 100% sensitivity and 100% specificity with good inter-rater reliability over a clinically relevant range of EG concentrations (27 to 281 mg/dl). Our experience using the REACT EGT kit found the assay universally positive regardless of the presence of EG. The PPV of 0.45 implies that fully 55% of positive results in our study population (where the prevalence of confirmed EG exposure=0.44) are false positives. An increase in the prevalence of EG exposure among suspected cases would improve the PPV of the test, though any increase in PPV would simply mirror the increase in prevalence owing to the almost universal positivity of the Kit irrespective of the presence of EG in the sample. This may have been a result of trivial to moderate concentrations of PG found in nearly half our study sample (31/56). Nonetheless, the extremely low specificity makes the REACT EGT kit unsuited for clinical application. In addition, even an extremely accurate qualitative EG assay would still necessitate quantitative confirmation of positive results in order to guide therapy. A highly specific qualitative assay might be sufficient to rule out EG exposure, with considerable savings of antidotal and other healthcare resources. In such a case the test kit would be analogous to a urine pregnancy test and the trivial cost of the EGT kit would be a fraction of each empiric course of EG therapy avoided. As it stands, the question of future applicability of the veterinary test kit is unlikely to be answered: the manufacturer ceased production of the REACT EGT kit towards the end of our study. Remaining veterinary wholesaler supplies have by now been exhausted.

ZBx LQ Ethylene Glycol Assay Performance

The ZBx LQ Ethylene Glycol assay had none of the limitations we observed with the REACT kit. Evidence from the analysis of 77 samples (33 positive for EG) by the manufacturer found a LOD of 5 mg/dl and a LOQ of 12 mg/dl with 100% sensitivity and 100% specificity. Previous method comparison of the assay with GC/MS by the manufacturer, calculated by correlation with linear regression, provided a best fit of y=1.0227x-1.24 and a correlation coefficient of 0.9725.[9]

In our study, analysis of 35 clinical samples (EG positive=11; range of EG concentrations from 6.3 to 620 mg/dl) also found the assay performed with a LOD lower than our lowest GC/MS positive sample (6.3 mg/dl) with 100% sensitivity and 100% specificity. In addition to a very robust sensitivity/specificity, the assay has a clinically relevant LOD. Our study did not evaluate LOQ for our 11 positive samples, however, our two lowest confirmed EG values – 6.3 mg/dl and 6.9 mg/dl – had corresponding ZBx LQ Ethylene Glycol Assay quantitative concentrations of 9.9 and 7.4 mg/dl respectively. Correlation between GC/MS derived concentration and quantitative ZBx LQ Ethylene Glycol Assay result was very robust (r=0.9803) and similar to the correlation found by the manufacturer when performance testing the assay (r=0.9725). Despite the wide range of EG concentrations in our 11 positive samples, the greatest difference occurred with our highest observed value (630 mg/dl by GC/MS); the corresponding ZBx LQ Ethylene Glycol Assay quantitative result was 423 mg/dl.

The ZBx LQ Ethylene Glycol Assay performed with a LOD sufficient for the rapid identification of clinically relevant EG exposures. Human risk assessment of EG has demonstrated that the human No Observed Adverse Effect Level (NOAEL) of EG is 150 mg/kg.[12] A 2 year daily oral dose bioassay (rat) found a NOAEL of 200 mg/kg. A 5 mg/dl LOD/12 mg/dl LOQ is sufficient to identify clinically relevant exposure to EG which result in a serum concentration of 25 mg/dl or higher. Such patients require intervention with antidotal therapy or enhanced elimination which

is in agreement with the current practice among the nation's medical toxicologist and cited in the criterion standard clinical reference text.[13]

Limitations

Our analysis using the ZBx LQ Ethylene Glycol Assay was limited by a small sample size. As our study relied on analysis of serum samples collected for clinical purposes, the volume we received for each enrolled participant in the study varied greatly. Only 40 ul was required for the REACT EGT kit, and 2 ml were required for confirmatory EG, PG and volatiles analysis at the reference laboratory. Unfortunately, only 35 samples of sufficient remaining volume (250 ul) were received to permit analysis in duplicate with the ZBx LQ Ethylene Glycol Assay (this included 18 samples which had sufficient volume after REACT EGT kit analysis).

In addition, lack of observer blinding to enrolled patient exposure status when interpreting the color change of the REACT EGT kit is a potential source of bias. It is unlikely that a further, follow-on study of the REACT kit is necessary given its lack of specificity. If the assay is redesigned or a similar POC qualitative colorimetric EG test kit is studied in the future, observers blinded to case status – and who are not part of the study team - should be used to interpret test kit positivity.

Cost Effectiveness

The ZBx LQ Ethylene Glycol Assay has recently completed development and may revolutionize the care of suspected EG poisoned patients. The assay is approved for clinical use in Canada and Europe, and has approval as a Research Use Only test in the US; the manufacturer is in the process of FDA 510k filing. Lack of a readily available hospital clinical assay to establish or rule out EG exposure has led to excessive healthcare costs, expenditure of rare healthcare

resources (antidote, dialysis capacity, critical beds), and unnecessary morbidity and mortality for patients.

If the ZBx LQ Ethylene Glycol assay performance measures are validated in a large, pivotal study and FDA approval for clinical use is obtained, the benefits of this technology to the US healthcare sector would be significant. No current comparable technology exists. A conservative estimate of the number of GC/MS EG analyses requested by clinicians in the U.S. each year for suspecting EG poisoning, and sent to a reference laboratory, is 2 times the approximately 5000 EG exposed patients reported to AAPCC annually.[1] This estimate of 10,000 tests for EG exposure is based on the work of Sutter, et al, which determined that 44% of acidemic patients in whom the diagnosis of EG exposure was considered actually leave the hospital with a confirmed diagnosis of EG (or methanol) exposure. In addition, EG quantitative analysis may be performed more than once on an exposed patient in order to guide therapy, plan additional antidote administration, or determine an ongoing need for dialysis. In addition to the cost savings for avoided reference laboratory testing and unnecessary antidote administration for 5,000 clinically suspected but unexposed patients, there would be better stewardship of hospital resources such as ICU beds.

Follow-Up Studies and Cost Effectiveness Research

One major limitation to our study is the small sample size and lack of power calculation for an investigator chosen minimum diagnostic accuracy of the REACT and ZBx LQ tests. Studies of diagnostic test performance will benefit from an appropriate sample size adequate to assure the statistical significance of the performance measure (sensitivity; specificity) being evaluated. Sample size calculations are universally performed in clinical trials, but are infrequently performed in studies of new diagnostic tests. A future pivotal study of the ZBx LQ EG assay might estimate a minimum sample size using the method of Flahault, et al.[14] Briefly, this

method allows selection of an hypothesized sensitivity (specificity) and an investigator-specified lower 95% confidence interval of test sensitivity (specificity) which is violated with a <5% probability. Tables provide case (control) sample size for the desired sensitivity (specificity) and the minimum acceptable lower 95% CI for sensitivity (specificity) selected. The non-investigator-specified N is then calculated based on prevalence of disease (EG exposure), using the equation $N_{cases}=N_{controls}[(1\text{-prev/prev})]$. As a screening test, further studies of the ZBx LQ assay might make sample size calculations based on investigator-specified specificity estimates. A robust 0.99 specificity with a lower 95% CI for specificity of 0.98 would require a control sample size of 1567 using the tables provided by Flahault. Sutter, et al, reported a prevalence of 0.44 of EG exposure in patients suspected of EG toxicosis and reported to a regional poison control center.[10] Using the equation for N_{cases} provided by Flahault, a 0.44 prevalence of EG exposure in suspected EG intoxicated patients - and a control sample size of 1567 - yields a case sample size of 1994 for a total study sample of 3561.

As previously mentioned, current ethylene glycol testing methods lead to unnecessary critical care admissions and expensive antidotal care. Because of the current delays in result reporting, many patients receive several doses of fomepizole in critical care settings before clinicians learn whether or not risk of toxicity exists. Sutter et al. reported that only 44% of acidemic patients with a history of potential EG ingestion are actually poisoned.[10] The frequency of confirmed EG ingestion among 56 subjects enrolled during our study was 0.39. Availability of a rapid, discriminating ethylene glycol assay with result reporting available within 30 minutes of sample collection would avoid considerable unnecessary resource utilization.

A future pivotal study design might include estimation of the potential reduction in healthcare spending the ZBx LQ EG assay would have on patients who are diagnosed as unexposed. Potential healthcare spending reduction could be estimated in one of two ways.[15,16]

For patients correctly identified as nontoxic by the ZBx LQ assay, the difference in charges could be estimated using Centers for Medicare & Medicaid (CMS) Strategic Planning estimates based on the year of data analysis using the formula below. The values for mean charge for a hospital stay, and for fomepizole administration, are examples based on current CMS policy:

(LOS†)($6770‡/24 hours) + (Dose of fomepizole received)($7.072‡/15 mg)

†Length of stay is the time from ED arrival to time of hospital discharge, rounded to the nearest whole hour.

‡Value should be adjusted for inflation based on CPI for calendar year 201X if available CMS rate is not current

This formula will demonstrate an estimate of charge using standard CMS policy and will be useful to parties interested in reducing the economic burden incurred by patients and government as a result of improved diagnostic testing.

Alternately, healthcare entities may be more interested in savings for hospitals. Although reducing length of stay is important, inpatient manpower and operating expenses tend to be fixed costs. Hospital resource utilization specialists, as well as treating medical toxicologists, may be more interested in savings from better stewardship of antidotes such as fomepazole. Individual hospital costs vary according to contracts with manufacturers; the average cost for a 1.5 g vial of fomepizole is $1000 in 2010 dollars. This confers an average cost for patients weighing 50, 70, and 100 kg of $500, $700, and $1000 for the first dose, respectively. Interestingly, the charge conferred to patients may be considerably different; the current Centers for Medicaid & Medicare Services payment limit is $6.403 per 15 mg. This discrepancy means hospitals lose, on average, about 35 cents for every $1 spent on fomepizole when treating Medicaid/Medicare patients. In non-exposed patients who are successfully identified by the ZBx LQ EG assay, calculated actual cost savings from improved antidote utilization is as follows:

(Total dose of fomepizole received in mg)($1000‡*/1500 mg)

‡Value adjusted for inflation based on CPI for calendar year 201X if available CMS rate is not current

*Actual value used for cost calculation will vary among participating hospitals

CONCLUSION

We conducted an un-blinded, cross sectional study of a veterinary, POC qualitative assay and a clinical chemistry analyzer-based quantitative assay on patients suspected to have EG exposure. The REACT EGT kit had near-zero specificity making it an ineffective test for the rapid diagnosis of EG toxicosis. The ZBx LQ EG assay demonstrated high sensitivity and specificity and a robust correlation with criterion standard testing (GC/MS) for the diagnosis of EG exposure. In addition, the assay has a clinically appropriate LOD for both the diagnosis of EG intoxication, and the quantitative nature of the test could be used to guide (and terminate) subsequent management in poisoned patients. The results of our study suggest a larger, pivotal investigation of the assay, appropriately powered to validate the 100% specificity and high correlation with GC/MS we found, is warranted.

The availability of timely clinical laboratory diagnosis of EG exposure would vastly improve the care of potentially EG poisoned patients. On the healthcare economics side, the ability to reliably rule-out EG poisoning in patients where there is a clinical suspicion, but no unequivocal exposure history to EG, would lead to considerable savings of expensive and limited antidote, avoidance of high-acuity (ICU) admission to the hospital, and possibly prevent the unnecessary institution of hemodialysis in ultimately unexposed patients.

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Figure 1 – Patient Enrollment

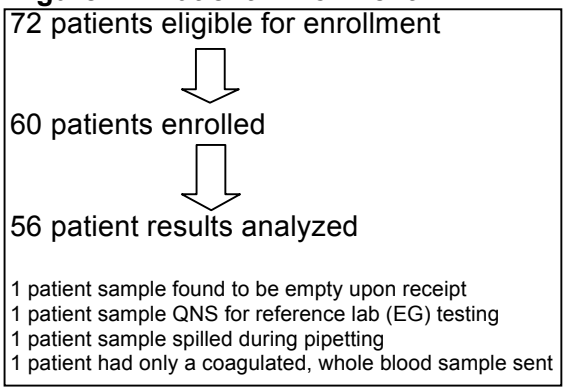

Figure 2 – Linear Regression and Correlation of GC/MS EG (mg/dl):ZBx LQ EG (mg/dl)

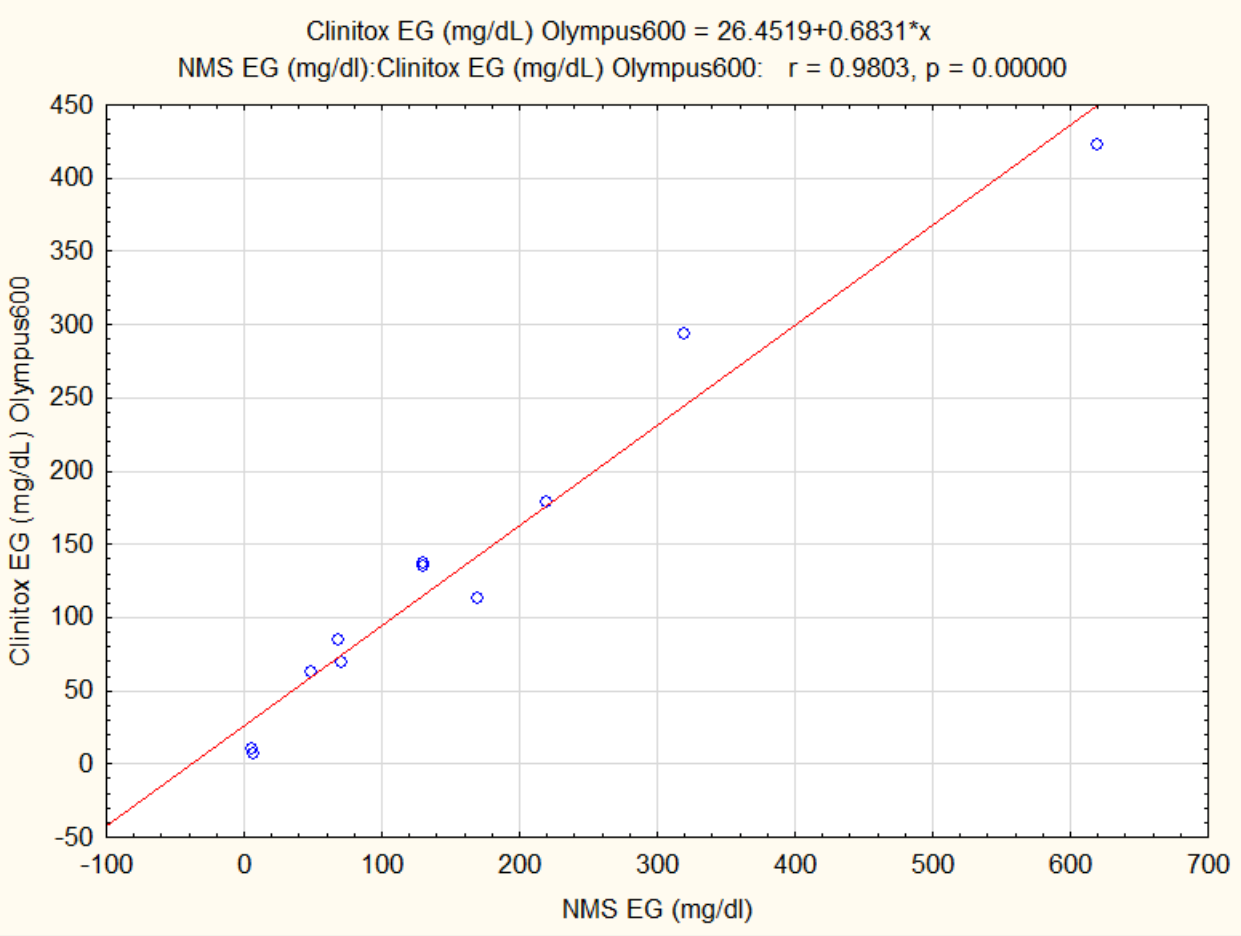

Table 1 – Summary statistics stratified over case status

	NMS EG (mg/dl)															
	Not Detected/Negative (N=34)					Detected/Positive (N=22)					Total (N=56)					p value (Negative vs Positive)
	N	Sum	Mean	Std. Deviation	Frequency (%)	N	Sum	Mean	Std. Deviation	Frequency (%)	N	Sum	Mean	Std. Deviation	Frequency (%)	
Age	34	1491	43.85	16.70		22	949	43.14	13.89		56	2440	43.57	15.53		0.87
Cr	34	81	2.38	4.02		22	36	1.64	1.36		56	117	2.09	3.25		0.41
Bicarb	32	643	20.09	7.38		20	319	15.95	7.46		52	962	18.50	7.61		0.56
EtOH	6	245	40.83	32.15		4	108	27.00	14.00		10	353	35.30	26.28		0.45
Osm	13	4111	316.23	29.70		16	5503	343.94	37.65		29	9614	331.52	36.52		0.04
Osm gap	11	275	25.00	22.39		15	706	47.07	31.57		26	981	37.73	29.70		0.06
AG	28	467	16.68	10.26		18	338	18.78	8.19		46	805	17.50	9.47		0.47
Gender																0.66
Female					8 (22.2)					6 (27.3)					14 (24.1)	
Male					28 (77.8)					16 (72.7)					44 (75.9)	
EtOHStatus																0.78
Not detected					24 (80.0)					13 (76.5)					37 (78.7)	
Detected					6 (20.0)					4 (23.5)					10 (21.3)	

Table 2 – Summary statistics stratified over study status

	Patient type															p value (Refused vs Enrolled)
	Refused (N=12)					Enrolled (N=56)					Total (N=68)					
	N	Sum	Mean	Std. Deviation	Frequency (%)	N	Sum	Mean	Std. Deviation	Frequency (%)	N	Sum	Mean	Std. Deviation	Frequency (%)	
Age	12	423	35.25	10.72		56	2440	43.57	15.53		68	2863	42.10	15.07		0.08
Cr	9	10	1.11	.33		56	117	2.09	3.25		65	127	1.95	3.03		0.37
Bicarb	9	160	17.78	8.39		52	962	18.50	7.61		61	1122	18.39	7.66		0.80
EtOH	5	230	46.00	25.47		10	353	35.30	26.28		15	583	38.87	25.62		0.47
Osm	8	2777	347.13	34.56		29	9614	331.52	36.52		37	12391	334.89	36.22		0.29
Osm gap	6	197	32.83	25.04		26	981	37.73	29.70		32	1178	36.81	28.57		0.71
AG	6	107	17.83	9.24		46	805	17.50	9.47		52	912	17.54	9.35		0.94
Gender																0.51
Female					4 (33.3)					14 (24.1)					18 (25.7)	
Male					8 (66.7)					44 (75.9)					52 (74.3)	
EtOHStatus																0.02
Notdetected					3 (37.5)					37 (78.7)					40 (72.7)	
Detected					5 (62.5)					10 (21.3)					15 (27.3)	

Table 3 – Sensitivity/specificity of the REACT EGT kit versus exposure status

		EG Exposure Status		Total
		Not Detected/Negative	Detected/Positive	
EGT Kit Result	Negative	1	0	1
	Positive	25	15	40
Total		26	15	41

Sensitivity = 1.00; Specificity = 0.04; Positive Predictive Value = 0.45; Negative Predictive Value = 0.55

Table 4 – Inter-rater reliability (κ) of Observer 1 and Observer 2 for the REACT EGT kit

		Observer 2		Total
		Negative	Positive	
Observer 1	Negative	1	0	1
	Positive	3	37	40
Total		4	37	41

$\kappa = 0.38$; $p < 0.05$

Table 5 – Sensitivity/Specificity of the ZBx LQ Ethylene Glycol Assay

		EG Exposure Status		Total
		Not Detected/Negative	Detected/Positive	
ZBx EG	Not Detected/Negative	24	0	24
	Detected/Positive	0	11	11
Total		24	11	35
Sensitivity = 1.00; Specificity = 1.00				

Table 6 – Sensitivity/specificity of the REACT EGT kit stratified over propylene glycol status

		EG Exposure Status		Total
		Not Detected/Negative	Detected/Positive	
PG = Not Detected/Negative				
EGT Kit Result	Negative	1	0	1
	Positive	9	8	17
Total		10	8	18
Sensitivity = 1.00; Specificity = 0.10				
PG = Detected/Positive				
EGT Kit Result	Negative	0	0	0
	Positive	16	7	23
Total		16	7	23
Sensitivity = 1.00; Specificity = 0.00				