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Comparative analysis of hospital and forensic laboratory ethanol concentrations: A 15 month investigation of antemortem specimens

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ABSTRACT

Quantitative serum alcohol concentrations from regional hospitals (from specimens collected at time of hospital admission) were compared to results from whole blood (from specimens collected at the time of hospital admission) concentrations measured at the San Diego County Medical Examiner's Office (SDCMEO). Over a 15 month period (January 2012 to March 2013), the postmortem forensic toxicology laboratory analyzed a total of 2,321 cases. Of these, 280 were hospital cases (antemortem) representing 12% of the overall Medical Examiner toxicology casework. 59 of the 280 hospital cases (or 21%) screened positive for alcohol (ethanol). 39 of these 59 cases were included in the study based on available specimens for quantitative analyses. This investigation indicated that serum hospital ethanol concentrations correlated well ($R^2 = 0.942$) with ethanol values determined at SDCMEO (generally measured in whole blood). There was an observed negative bias with an average of −14.1%. A paired t-test was applied to the data and it was shown that this observed bias is statistically significant. These differences in ethanol concentrations could result from differences in specimen, analytical techniques, and/or calibration. The potential for specimen contamination is also discussed.

1. Introduction

Ethanol (alcohol) is the most common quantitative drug test ordered in both clinical and forensic toxicology.[1,2] The quantitation of ethanol in biological specimens can help determine cause and manner of death.[2] The accuracy of the ethanol quantitation is especially important in forensic toxicology settings. There are multiple analytical methods for ethanol quantitation, which may introduce variation in the measured ethanol value. Within sample variation may also be attributed to the time frame between the two separate analyses. In such instances, the true ethanol concentration may vary since as the sample ages, losses of ethanol due to evaporation can become significant.[3] Other causes for variation in the ethanol values come from systematic biases between the two different analytical methods. This is especially true when comparing an enzymatic assay, commonly used in the hospital setting, with a chromatographic ethanol assay used in forensic settings. Enzymatic assays do not measure the concentration of ethanol directly, instead, they measure an absorbance change caused by the production of NADH which is then related back to the concentration of ethanol.[4] Unfortunately, some small molecules oxidized by their respective enzymes can also produce NADH, therefore possibly increasing the perceived concentration of ethanol.[5,6] Calibration differences between methods may also play a role in the variability of quantitation between different methods.

This study compared the ethanol quantitation values of 39 ethanol positive cases, where samples in the same collection set were analyzed first at the hospital of origin and then again at the San Diego County Medical Examiner's Office (SDCMEO). Ethanol quantitation differences and contributing factors which may have caused them are discussed.

2. Methods

2.1. Inclusion criteria

Specimens included in this ethanol correlation study were collected over 15 months (January 2012 to May 2013). Of the 2,321 cases for which toxicological analysis was performed, 280 were hospital cases (antemortem) representing 12% of the overall Medical Examiner toxicology casework. Ethanol positive cases were

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initially quantified at the hospital of arrival from samples collected before the death of the patient. If the patient death became a medical examiners case, ethanol values were analyzed at the SDCMEO as part of routine casework. Samples were measured at SDCMEO using the in-house ethanol screening and quantitation methods. 59 of the 280 hospital cases screened positive for alcohol (ethanol). From these 59 cases, 39 had sufficient and appropriate material (original whole blood or serum) for ethanol quantitation at SDCMEO. The majority of antemortem whole blood samples received at the medical examiner's office from hospitals were in EDTA lavender top tubes. Other tube types such as sodium citrate and sodium fluoride were also received and tested. All samples, upon arrival at SDCMEO were stored at 4 °C until analyzed. The maximum delay between testing at the hospital and the SDCMEO was ten days. A paired t-test was used to evaluate differences between the concentrations of ethanol determined by the different techniques with a p value <0.05 considered statistically significant.

2.2. Hospital ethanol quantitation

Most hospitals use an enzymatic assay to measure concentration of ethanol in plasma or serum. This enzymatic assay contains a known quantity of the enzyme alcohol dehydrogenase. Oxidation of ethanol by this enzyme generates a stoichiometric amount of NADH, the rate of which is monitored spectrophotometrically at 340 nm.[4]

2.3. Ethanol quantification (SDCMEO)

2.3.1. Chemicals and apparatus

The internal standard n-propanol was reagent grade (Burdick & Jackson) and was purchased from VWR (Radnor, PA). The methanol, isopropanol and acetone standards were OmniSolv grade and were also purchased from VWR. Ultra high purity zero water was obtained from Pall Corporation “Cascada” system. Potassium dichromate was AR Primary standard grade and was purchased from NIST (Gaithersburg, MD). Sodium thiosulfate, potassium iodide, and soluble starch were purchased from Mallinckrodt (St. Louis, MO). Sulfuric acid was purchased from Fisher Scientific (Waltham, MA).

200 proof ethanol was un-denatured, USP grade and was purchased from Spectrum Chemical Corporation (Gardena, CA). Aqueous stock internal standard solutions containing 0.05% n-propanol in deionized water and working internal standards containing 0.00625% n-propanol in deionized water were prepared using calibrated volumetric pipettes. Three calibrators consisting of secondary alcohol standards were created using the following method. Approximate ethanol concentrations of 0.10 g/dL, 0.20 g/dL, and 0.30 g/dL were prepared by volumetric addition of 200 proof ethanol to deionized water. Each of the calibrator's exact concentration was then determined by using a direct oxidimetric method. This method uses a primary standard of potassium dichromate in sulfuric acid. The primary standard of potassium dichromate is traceable to National Institute of Standards and Technology (NIST) potassium dichromate standard. Solutions of exactly 0.1304 N potassium dichromate were made by weighing previously desiccated potassium dichromate using a Mettler AG104 analytical balance followed by dissolution with deionized water in a volumetric flask. Addition of a known quantity of excess of potassium dichromate was used to ensure all ethanol in a sample will be oxidized. After reaction completion, the concentration of the remaining potassium dichromate was determined by addition of potassium iodide and back titration of the produced iodine with sodium thiosulfate. Back calculation of the consumed concentration of sodium thiosulfate yielded the amount of unreacted potassium dichromate. Subtraction of the potassium dichromate's known initial concentration by the unreacted concentration yielded the amount of potassium dichromate consumed which stoichiometrically gives the concentration of ethanol in the solution. The concentration of each secondary alcohol calibrator from the aforementioned method was determined by averaging six replicate measurements. A volatile reference solution (VRS) was prepared by diluting 0.5 mL methanol, 1.0 mL ethanol, 1.0 mL isopropanol and 0.5 mL acetone to 1000 mL with deionized water. This VRS was analyzed with each batch of casework to confirm the accuracy of the alcohol retention times. Two commercial whole-blood toxicology controls containing 0.081 g/dL and 0.202 g/dL of ethanol in whole blood were obtained from Cliniqa Corporation. (San Marcos, CA), and an in-house negative control prepared with only diluent and internal standard (n-propanol) were run with each batch of calibrators and casework.

2.3.2. Specimen preparation

Ethanol and other volatiles were analyzed using a GC-FID-Headspace procedure. 50 μL of calibrator standards, controls or samples (whole blood or serum) were added to individual headspace auto-sampler vials. 2.0 mL of the working internal standard solution containing 0.00625% n-propanol was then added to each vial. Samples were then crimp-capped and placed in the sampler tray for headspace GC analysis which were equilibrated at 40 °C. Samples were run in duplicate and their averages were reported.

2.3.3. Instrumentation

2.3.3.1. Ethanol screen (SDCMEO). Before the described quantification procedure was performed, all cases were initially determined to be positive for ethanol using a screening procedure. The screening method and instrumentation utilized were identical to the quantification procedure described, apart from the analytical column which was a RTX-BAC2 (Restek Technologies) (30 m, 0.32 mm diameter) column, and the method was calibrated using a single alcohol concentration of 0.20 g/dL.

2.3.3.2. Ethanol quantification (SDCMEO). Injections of headspace vapors were made onto a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a Model G1888 automatic headspace sampler and a flame-ionization detector. The GC column was a RTX-BAC1 (Restek Technologies) (30 m, 0.32 mm diameter) with hydrogen as the carrier gas. The GC oven was held steady at a temperature of 40 °C. The total chromatography time per injection was 3.5 min. Volatile identification was based on retention time and quantitation based on calibrated area ratios of the volatile and the internal standard (n-propanol). Using two columns to confirm the presence of ethanol provides higher confidence that the analysis is not subject to other volatile interferences. A list of common volatiles and their retention times on the columns used for screening and quantitation are shown in Table 1.

2.3.4. Accuracy and Precision

All calibrators were within 5% of the target concentration when they were back calculated. A calibration curve was constructed from all three calibrators. The calibration used a linear regression fit in which $R^2 \geq 0.99$. The limit of detection (LOD) was 0.005 g/dL, and the limit of quantification (LOQ) was 0.02 g/dL. The two positive control samples included in each batch were compared to the prepared values of 0.081 g/dL and 0.202 g/dL which back calculated to be within 5% of the target concentration. Accuracy, assessed over a nine month period, was 0.077 g/dL or 95% of the target (from 128 individual determinations) for the 0.081 g/dL control, and 0.198 g/dL, 98% or the target (from 80 individual determinations) for the 0.202 g/dL control. Precision (% coefficients of variation), over this

Table 1
GC retention times for common volatiles.

Volatile	Column retention time for screen (min)	Column retention time for quantification (min)
1,1 difluoroethane	0.649	0.780
Methanol	1.002	0.752
Ethanol	1.327	0.935
Acetone	1.433	1.386
Isopropanol	1.563	1.136
Internal standard (n-propanol)	2.509	1.517

same time frame was 2.2% and 1.5% for the 0.081 g/dL, and 0.202 g/dL controls respectively.

3. Results and discussion

Results from 39 ethanol positive specimens analyzed at both hospital and SDCMEO are shown in Table 2. The initial ethanol screen and quantitation at SDCMEO show excellent agreement with each other. All of the specimens analyzed at the hospital were serum, while only four of the 39 specimens analyzed at SDCMEO were serum. Fig. 1 shows the correlation of the ethanol quantitation values received from the hospital of origin compared to the ethanol quantitation analysis values determined at SDCMEO. Case 11 was an outlier with a hospital ethanol value of 0.34 g/dL as compared to the SDCMEO ethanol value of 0.20 g/dL. Given the magnitude of the difference, case 11 is most likely due to two different samples being analyzed. Although this discrepant case exists, regression analysis with its inclusion still showed good correlation with an $R^2 = 0.942$. Regression data with removal of case 11 showed excellent correlation with an $R^2 = 0.981$.

Fig. 2 displays a percent bias plot between SDCMEO and hospital ethanol concentration value. This plot showed a general negative bias between the SDCMEO values and the hospital's values with an average bias of −14.1% which is particularly obvious at the lower ethanol concentration range (<0.10 g/dL). A paired t-test applied to the two data sets (enzymatic ethanol value and GC ethanol value) was less than 0.0001 indicating a statistical difference. From an interpretive standpoint, the difference may not be large enough to cause concern except near the legally mandated BAC cutoff of 0.08 g/dL (legal limit for operating a motor vehicle in California). The clinical interpretation of the concentration of ethanol as determined by the hospital and by the SDCMEO is likely to be the same, since there is a large degree of variability in impairment at similar blood ethanol concentrations.[7]

A portion of the observed bias between SDCMEO and hospital labs is likely attributed to the use of whole blood at SDCMEO and serum in hospital lab analysis.[7] Whole blood, on average, will give a lower ethanol result than the use of serum (reported at 11% lower).[8] Additionally, the observed negative bias may be attributed to

Table 2
Ethanol concentration data of cases from hospital and SDCMEO assays.

Case	Date	Manner	Sample type (SDCMEO)	HOSP EtOH (g/dL)	SDCMEO EtOH screen (g/dL)	SDCMEO EtOH quant (g/dL)	Percent bias
1	1/2/2012	Suicide	Serum	0.263	0.257	0.256	−2.7
2	1/5/2012	Suicide	Whole blood	0.170	0.168	0.170	0.0
3	1/25/2012	Suicide	Whole blood	0.120	0.127	0.129	7.0
4	2/22/2012	Accident	Whole blood	0.036	0.029	0.029	−24.1
5	2/25/2012	Homicide	Whole blood	0.200	0.205	0.205	2.4
6	3/10/2012	Accident	Whole blood	0.280	0.301	0.291	3.8
7	3/30/2012	Suicide	Whole blood	0.041	0.033	0.033	−24.2
8	4/5/2012	Suicide	Whole blood	0.324	0.320	0.309	−4.9
9	4/30/2012	Natural	Whole blood	0.050	0.044	0.039	−28.2
10	5/8/2012	Accident	Whole blood	0.383	0.347	0.348	−10.1
11	5/17/2012	Accident	Serum	0.340	0.194	0.195	−74.4
12	5/20/2012	Natural	Whole blood	0.222	0.191	0.190	−16.8
13	6/1/2012	Natural	Whole blood	0.080	0.096	0.089	10.1
14	6/21/2012	Accident	Whole blood	0.089	0.064	0.062	−43.5
15	7/6/2012	Natural	Serum	0.165	0.149	0.149	−10.7
16	7/6/2012	Accident	Whole blood	0.256	0.226	0.223	−14.8
17	7/9/2012	Accident	Whole blood	0.250	0.233	0.222	−12.6
18	7/21/2012	Accident	Serum	0.245	0.231	0.227	−7.9
19	7/23/2012	Accident	Whole blood	0.353	0.328	0.313	−12.8
20	7/29/2012	Accident	Whole blood	0.116	0.086	0.088	−31.8
21	7/29/2012	Natural	Whole blood	0.090	0.064	0.066	−36.4
22	8/4/2012	Homicide	Whole blood	0.410	0.401	0.413	0.7
23	8/29/2012	Accident	Whole blood	0.060	0.055	0.055	−9.1
24	9/6/2012	Suicide	Whole blood	0.149	0.128	0.126	−18.3
25	9/12/2012	Accident	Whole blood	0.291	0.251	0.238	−22.3
26	9/16/2012	Homicide	Whole blood	0.168	0.142	0.138	−21.7
27	9/27/2012	Accident	Whole blood	0.140	0.130	0.129	−8.5
28	10/2/2012	Accident	Whole blood	0.220	0.209	0.209	−5.3
29	11/11/2012	Accident	Whole blood	0.176	0.154	0.155	−13.5
30	11/16/2012	Undetermined	Whole blood	0.360	0.374	0.378	4.8
31	11/14/2012	Accident	Whole blood	0.251	0.219	0.216	−16.2
32	11/22/2012	Accident	Whole blood	0.253	0.222	0.218	−16.1
33	12/3/2012	Accident	Whole blood	0.173	0.147	0.145	−19.3
34	12/9/2012	Accident	Whole blood	0.295	0.285	0.277	−6.5
35	12/12/2012	Accident	Whole blood	0.038	0.028	0.031	−22.6
36	1/3/2013	Accident	Whole blood	0.180	0.143	0.150	−20.0
37	2/7/2013	Accident	Whole blood	0.023	0.021	0.020	−15.0
38	2/17/2013	Accident	Whole blood	0.295	0.280	0.273	−8.1
39	2/24/2013	Accident	Whole blood	0.340	0.348	0.342	0.6

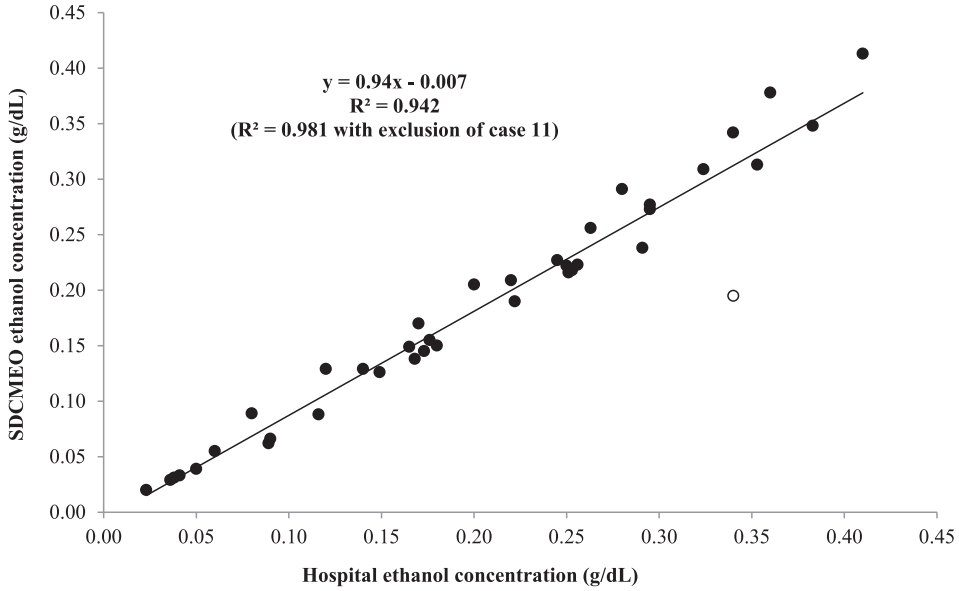

Fig. 1. Correlation of hospital and SDCMEO ethanol concentrations (case 11 marked as unfilled circle).

systemic bias related to differences in calibration between the two methods. In this cohort of specimens there are three serum specimens that were analyzed by the SDCMEO and the hospitals. In all three cases where serum was analyzed by both labs, the results agree within 10%.

During ethanol determination using the screening and quantitation methods, other volatiles such as low molecular weight alcohols and aromatics are also analyzed. Since these volatiles may have been ingested and contributed to the death of the individual, it is important to differentiate between true exposure and chemical contamination. Although none of the specimens in this cohort contained any volatiles other than ethanol determined by headspace GC-FID, there have been reports of specimens contaminated with isopropanol and toluene.[9]

Isopropanol or rubbing alcohol is a common antiseptic used to prepare the skin for venipuncture and to disinfect medical tools.[10] Like ethanol, consumption of isopropanol leads to inebriation effects making its presence in ED patients not uncommon. Isopropanol is metabolized to acetone[11] and consumption of this alcohol can cause gastrointestinal bleeding and severe hypotension leading to coma or death.[10] If a patient were to consume isopropanol, appreciable amounts of acetone would also be found via GC headspace assays. Therefore if isopropanol is found without a concurrent quantity of acetone, it can reasonably be concluded that

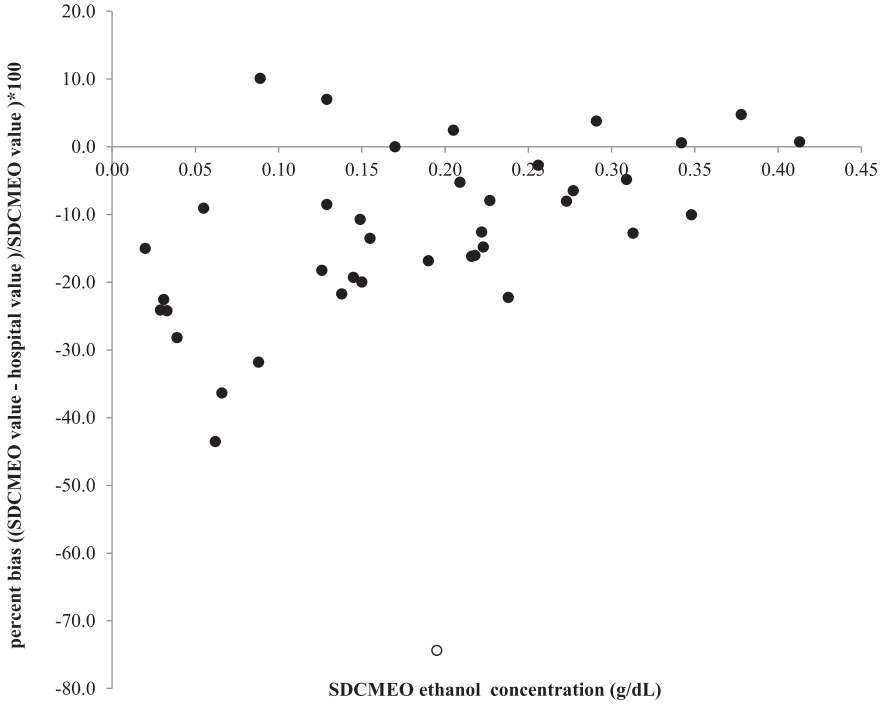

Fig. 2. Bias of SDCMEO vs. hospital ethanol concentrations (case 11 marked as unfilled circle).

the isopropanol in the specimen is due to ex vivo contamination. One such case occurred at SDCMEO in which an antemortem sample negative for ethanol, as reported by the originating hospital, contained 0.29 g/dL of isopropanol and no detectable acetone as measured by headspace GC-FID at SDCMEO. Analysis of a femoral whole blood sample from this decedent collected at SDCMEO revealed no isopropanol or acetone, indicating the first sample collected at the hospital was contaminated with isopropanol.

Toluene, an aromatic hydrocarbon, used in preparation of the gels for serum or plasma separator tubes has also been observed as a contaminant of blood collected in these kinds of specimen containers.[9] During routine ethanol quantitation at SDCMEO, it was discovered that an appreciable amount of toluene was recovered from plasma samples from some gel separator tubes. In particular, two cases of toluene contamination came from light blue sodium citrate plasma separator tubes. Although toluene contamination has been reported,[9] sources of specimens from hospitals for forensic testing can be scarce and when necessary, these specimens are an essential part of casework.

4. Limitations

Although all of the ethanol values from SDCMEO were completed in-house using carefully followed methods described above, the original ethanol data was collected from multiple hospital laboratories in southern California. Each hospital laboratory's procedures contribute their own unique variation due to differences in drawing methods (e.g. nurse drawing off a line or venous phlebotomy), blood tube type, and analytical methods. We also acknowledge the possibility that a sample was switched for another (specifically case 11), altering the data obtained.

5. Conclusions

In conclusion, over a 15 month period, antemortem specimens represented 12% of the total case work received and analyzed in the forensic toxicology laboratory at the SDCMEO. Generally, the use of such specimens eliminated questions about postmortem changes in ethanol concentrations, thereby increasing the accuracy of ethanol concentrations near the time of death.

Furthermore, from the antemortem specimens received, we present a retrospective correlation study of ethanol concentration values from local hospitals and SDCMEO. Concentration values of 39 ethanol positive cases from hospitals and the SDCMEO correlated well with an $R^2 = 0.942$. However, the SDCMEO ethanol determinations showed a negative bias with respect to the hospital ethanol values with an average of −14.1%. Larger percent biases were seen in ethanol concentrations of <0.10 g/dL. Although the clinical interpretation regarding impairment is likely to be the same regardless if the concentration of ethanol was measured in a hospital lab or the SDCMEO, there could be medicolegal ambiguities if the determination hinges on the application of a specific cutoff such as 0.08 g/dL that is applied to driving under the influence statues. The SDCMEO forensic toxicology laboratory follows all procedures and requirements (analytical methods described herein) for forensic whole blood alcohol analyses as detailed by California's Department of Health Title 17, which is not practiced at most hospital laboratories. The SDCMEO laboratory is also frequently monitored for accuracy and performance under the requirements of this law, and all toxicologists are certified as forensic alcohol analysts under Title 17. Although hospital alcohol analyses may be validated and controlled, they do not satisfy this legal requirement. Also, the possibility of a sample being switched for another reminds us that these clinical samples do not follow a chain of custody and therefore may not be suitable for forensic purposes. Hospital laboratories quality is ensured through good laboratory practices, quality control, routine proficiency testing and certification by CLIA and/or CAP. This work should draw attention to the potential for ethanol value bias between enzymatic and GC methodologies which is most evident at lower blood ethanol concentrations and provide understanding and explanation of that bias.

Conflicts of interest

The authors of this manuscript have no conflicts of interest.

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