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Measurement of Chemical Analytes in Vitreous Humor: Stability and Precision Studies

ABSTRACT: The analytic accuracy and precision for measurement of chemical analytes in vitreous humor (VH) are critical if results are to be used in forensic pathology. The purpose of our study was to demonstrate the stability and the reproducibility of VH sodium, potassium, chloride, glucose, urea nitrogen, acetone, and beta-hydroxybutyrate in specimens obtained from both eyes in medical examiner cases. We also compared with calculated VH osmolalities. Small but significant increases were observed in VH electrolyte concentrations in specimens refrigerated 6–12 months: sodium pre 144 mmol/L, post 151 mmol/L; potassium pre 12.0 mmol/L, post 12.8 mmol/L; chloride pre 121 mmol/L, post 123 mmol/L. No differences were observed between eyes, and within-day precision for all electrolyte measurements were excellent, (<1%). Frozen specimens showed significantly higher measured (439 mOsmol/kg) as compared with calculated osmolality (305 mOsmol/kg), with 1% within-day precision and no significance between eye variation for glucose and urea nitrogen. In 20 of 24 medical cases selected for possible ketoacidosis, measurement of beta-hydroxybutyrate concentrations appears to be a promising diagnostic biomarker for confirming suspected ketosis in medical examiner cases by means of VH analysis.

KEYWORDS: forensic science, vitreous humor, electrolytes, beta-hydroxybutyric acid, cause and manner of death

Vitreous humor (VH) is often an invaluable specimen in forensic pathology cases in the determination of the cause and manner of death (1). It is well established that the chemical composition of VH is more stable postmortem (PM) than either blood or CSF (1–3) and reflects antemortem plasma concentrations of sodium (Na), chloride (Cl), and urea nitrogen (UN) (1–4). Thus, vitreous humor is an ideal postmortem specimen for analysis of chemical analytes as compared with blood and tissue. This is a result of its relative acellularity and low susceptibility to rapid chemical changes and contamination, microbial or otherwise. Plasma and serum electrolytes show erratic and unpredictable postmortem variations and hence are unreliable for determination of a decedent's antemortem status (3). VH Na and Cl have been shown to be stable after death for at least 18–30 h (1,4). In addition, the equilibrium between VH and blood is useful in determining electrolyte abnormalities. In the early PM period, markedly increased or decreased VH Na and Cl concentrations have been found in hospital cases and reflected antemortem abnormalities. However, it has also been shown that the converse is not true, in that cases with hyponatremia as the cause of death may have normal VH Na levels (4).

Unlike blood and CSF, there is no practical means of establishing a normal range for VH analytes in healthy, nonpathologic human eyes. VH obtained from surgically removed eyes cannot be assumed to be "normal" because invariably, some pathologic condition or injury necessitated enucleation. Earlier reports of normal ranges are marred by a lack of reported data, poor statistical analyses, and poorly described methodologies (1,3). Varying methodologies used in analyte analysis (5) and sample manipulation (6) are also concerns in attempting to interpret proffered normal values in the early, original reports. Between-eye differences for VH Na and Cl have been studied and discrepant results have been documented (6). Again, data and methodologies were either not provided and/or sample manipulation, including dilution and centrifugation, led to what may be questionable results. The most recent review of 200 cases revealed inter-eye differences for Na of $\leq$8 mmol/L, with only one case differing by more than 5%. An explanation offered for large inter-eye and intra-eye differences was repeated freezing and thawing of vitreous humor (6).

The interpretative value of VH electrolyte is most useful in cases in which there is a lack of antemortem plasma values. In addition, VH measurements are useful in cases in which there has been no resuscitation. Examples include hypernatremic dehydration in infants who exhibit no morphologic findings and who present as suspected sudden infant death syndrome (SIDS) (7) or decedents with non-apparent dehydration secondary to gastrointestinal water loss (diarrhea) but who do not demonstrate diagnostic gross autopsy findings (8,9). Furthermore, there was a good correlation between postmortem VH Na concentrations and antemortem Na concentrations in children who had been diagnosed with antemortem hyper or hypo-natremia (10).

Even without an experimentally determined normal range, VH Na and Cl concentrations are currently used in forensic practice to assist in the diagnosis of antemortem states, including psychogenic polydipsia (10), acute dehydration (7) and excessive salt intake (11). In addition, glucose is also useful in identifying hyperglycemia; determinations in over 6,000 VH specimens found no non-diabetics with glucose concentrations >200 mg/dL (2).

The primary purpose of the current study was to evaluate the stability and reproducibility of refrigerated VH specimens (from both eyes) for the measurement of Na, Cl, and K over a period of 6 to 12 months. A secondary purpose was to compare

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beta-hydroxybutyrate, acetone, and acetoacetate concentrations. A third was to examine calculated vs. measured osmolality concentrations in a subset of frozen VH specimens. Overall, these efforts validated the reliability of specific methodologies for the measurement of several VH chemical analytes. Results are expressed for both eyes as VH A and VH B specimens, as specimens were not specifically identified right and left eye at collection.

Materials and Methods

VH specimens were been obtained at autopsy from 126 medical examiner cases between 2001 and 2002. All protocols were approved by the local institutional review board. Some samples were stored frozen at −20°C ($n = 54$) or refrigerated at 4°C ($n = 48$) for 6 to 12 months. All vitreous samples were filtered prior to analysis. Briefly, VH specimens were filtered using the Amicon filter system, spinning for 20 min at 3500 rpm prior to analysis. The electrolytes, Na, K, and Cl (all mmol/L) were quantitated (minimum total sample volume 100 μL) via ion selective electrodes (Hitachi 911 Automatic Analyzer, Boehringer Mannheim, Indianapolis, IN; indirect potentiometry). Acetone (mg/dL) was measured by gas chromatography (Hewlett-Packard (HP) Series II 5890, Hewlett-Packard Co., Palo Alto, CA). Briefly, 200 μL of sample was pipeted along with 200 μL of a 200 mg/dL n-propanol internal standard into a 20 mL headspace vial. Vials were crimp sealed and incubated at 60°C prior to headspace autoinjection (HP 7694 Headspace sampler) onto a HP Model 5890 Capillary Gas Chromatograph, using a 30 meter SB-wax 530 micron column, injection temperature 250°C, oven temperature 55°C. The minimum detectable concentration was 5 mg/dL. Biorad Liquichek volatile controls were used for appropriate quality control, with 5.2% total imprecision (%CV) at 5.3 mg/dL. Acetoacetate was measured qualitatively by Acetest tablets (Bayer Corporation, Elkhart, IN; minimum sample volume three drops). Daily qualitative quality control was performed and found to be acceptable. Beta-hydroxybutyrate was measured in singlet using the blood beta-ketone test strip provided by MediSense on the Precision Xtra meter (Abbott Laboratories, MediSense Products, Bedford, MA) (minimum sample volume 1 drop). Daily quality control demonstrated a total %CV < 3% at 0.4 mmol/L and 4.1 mmol/L. Specimens were run in triplicate to establish internal precision for Na, Cl, and K measurements. Daily quality controls demonstrated a total %CV of <5% at the following respective concentrations: 140 mmol/L, 101 mmol/L, 3.4 mmol/L. Osmolality measurements (mOsm/kg H_2O) were performed by freezing point depression (Advanced Micro Osmometer Model 3300, Advanced Instruments, Inc., Norwood, MA) in single determinations (minimum sample volume 20 μL). Daily quality control demonstrated a total %CV < 3% at 291 mOsm/kg H_2O and 501 mOsm/kg H_2O. Urea nitrogen and glucose measurements were also performed in singlet (Hitachi 917; minimum sample volume 50 μL) and used as the basis for the calculation of the calculated osmolality as follows: 2 × Na (mmol/L) + glucose (mg/dL)/18 + UN (mg/dL)/2.8. Daily quality controls demonstrated a total %CV < 3.5% for glucose at 122 mg/dL and urea nitrogen at 18 mg/dL.

Statistical analyses were performed (SPSS, Mac v10, software) using paired-t tests and 2-way ANOVA. Significance was set at $p < 0.05$. No specimens were excluded because of insufficient sample volumes. Valid results were obtained and used for statistical analyses for all measured values.

Results

Table 1 (which shows mean and 95% confidence interval values) demonstrates that following storage at 4°C, small but statistically significant ($p < 0.006$) increases were observed in refrigerated ($n = 48$) specimens: Na pre-storage, 144 mmol/L − post-storage, 151 mmol/L; K pre-storage, 12.0 mmol/L − post-storage 12.8 mmol/L; Cl pre-storage, 121 mmol/L, post-storage, 123 mmol/L. Insignificant differences in concentration were found between eyes whether stored refrigerated (4°C) ($n = 48$) or frozen (−20°C) ($n = 54$) as shown in Table 2: Na < 2%; Cl < 3%; K < 4%. Within-day precision demonstrated <1% CV for all three electrolytes (Na, K, Cl), independent of storage conditions.

TABLE 1—*Comparison of vitreous humor electrolytes (*n = *48) stored refrigerated for 6 to 12 months.*

Electrolyte (mmol/L)	Specimen	Mean	95%CI	*P* value
Na	Original	144.6	142.9, 146.4	<0.001
	Mean	151.3	149.3, 153.3	
K	Original	12.0	10.5, 13.5	<0.001
	Mean	12.8	11.3, 14.4	
Cl	Original	121.1	118.9, 123.2	<0.001
	Mean	123.4	121.3, 125.5	

Original = initial concentration measured post autopsy; Mean = mean of triplet following storage; CI = confidence interval; *p* value by paired t-test.

TABLE 2—*Comparison of vitreous humor chemistry analytes (*n = *54) stored frozen over 6 to 12 months.*

Analyte	Specimen	Mean	95%CI	*P* value
Na, mmol/L	VH-A*	152.2	148.5, 155.8	0.42
	VH-B*	152.4	148.7, 156.0	
K, mmol/L	VH-A*	11.9	10.5, 13.3	0.73
	VH-B*	11.9	10.5, 13.3	
Cl, mmol/L	VH-A	127.9	124.3, 131.5	0.16
	VH-B	127.9	124.3, 131.4	
Glu, mg/dL	VH-A*	39.2	0.0, 96.7	0.08
	VH-B*	40.1	0.0, 97.9	
Osmol-meas	VH-A	439.2	416.7, 461.6	0.51
mOsmol/kg	VH-B	433.6	405.9, 461.2	
Osmol-calc	VH-A	305.9	287.0, 324.7	0.23
mOsmol/kg	VH-B	307.7	288.9, 326.4	
BUN, mg/dL	VH-A*	19.7	9.4, 30.0	0.31
	VH-B*	19.6	9.3, 29.9	

* Mean of 3 reps; Osmol = osmolality; meas (measured) and calc (calculated); *P* value by paired t-test; A and B represent the two different eyes.

For specimens stored frozen, Table 2 shows that VH calculated osmolality was lower than measured osmolality, 305 mOsmol/kg v. 439 mOsmol/kg, respectively ($p < 0.001$); independent of which eye ($n = 54$) was used. The measured osmolality showed <1% within-day precision. Between eye differences and within-day precision for VH glucose and urea nitrogen were <8% and <2%, respectively. Refrigerated specimens were not tested for osmolality.

Table 3 summarizes the findings for beta-hydroxybutyrate (both eyes), and acetone and acetoacetate (one eye), along with the medical examiner's recorded cause of death in 24 cases selected for possible ketoacidosis. Twenty of 24 cases (83.3%) demonstrated increased VH beta-hydroxybutyrate concentrations (>0.6 mmol/L). There were no significant differences between eyes. In contrast, no acetone was found in any case. Only 1 case (#9) showed a trace acetoacetate, which coincided with the highest beta-hydroxybutyrate concentration (5.9 mmol/L).

Discussion

The findings of this current study confirm the reliability of the Hitachi system for VH analyte determinations. We found no

TABLE 3—*Keto-acid concentrations and cause of death in 24 medical examiner cases.*

Case	BHB-A	BHB-B	Acetone	Acetest	Cause of Death
1	3.3	1.9	neg	neg	Diabetes mellitus type II
2	1.6	2.2	neg	neg	Alcoholism, ethanol <0.01 g/dL
3	2.9	2.9	neg	neg	Alcoholism, ethanol <0.01 g/dL
4	1.7	1.5	neg	neg	Ovarian cancer
5	1.8	1.8	neg	neg	Heart disease
6	2.1	1.4	neg	neg	Motor vehicle collision
7	1.7	2	neg	neg	Motor vehicle collision, opiates present
8	3.7	2.6	neg	neg	Myocardial infarction
9	5.9	6	neg	trace	Hypothermia, ethanol 0.18 g/dL
10	2.1	1.8	neg	neg	Hanging
11	3.3	1.9	neg	neg	Diabetes mellitus type II
12	2	2.8	neg	neg	Motor vehicle collision; myocardial infarction
13	1.2	1.8	neg	neg	In hospital death
14	2.6	2.6	neg	neg	Endocarditis
15	0.8	0.7	neg	neg	Ischemic heart disease
16	3.2	3	neg	neg	Myocardial infarction
17	3.4	3.4	neg	neg	Cardiomyopathy
18	2	2.3	neg	neg	Myocardial infarction
19	2.2	1.5	neg	neg	Diabetes mellitus type II; glucose 900 mg/dL
20	1.5	1.3	neg	neg	Diabetes mellitus type II; heart disease
21	0.3	0.2	neg	neg	Alzheimers
22	0.3	0	neg	neg	Hanging
23	0	0	neg	neg	Hypertension; diabetes mellitus type II
24	0	0	neg	neg	Insulin dependent diabetes; glucose 300 mg/dL

BHB-A,B = beta-hydroxybutyrate concentration (mmol/L) in eye A or eye B; neg = negative.

significant difference in triplicate sample analysis with a %CV for all VH electrolytes of <1%, without differences between eyes. Further, we conclude that beta-hydroxybutyrate appeared to be a sensitive marker for ketosis. Lastly, we also document substantial differences between calculated and measured osmolality, independent of which eye might be sampled.

Electrolyte determinations in VH specimens are frequently utilized in forensic cases to assist in the determinations of both cause and manner of death. VH is more stable after death than CSF or blood (1,2) and reflects antemortem electrolyte and chemical constituent status (2–4).

This study appears to be the first to examine the stability of refrigerated (4°C) VH samples over an extended time period (6–12 months). Refrigerated specimens showed small but statistically significant ($p < 0.006$) increases for Na, K, and Cl. However, inter-eye differences were not observed for either refrigerated or frozen and thawed samples. Further, no difference over time would have changed the medical examiner's interpretation of the findings. The correlation between eyes supports the work of Pounder et al., who studied between-eye differences of Na, Cl, and K (6). Utilizing fresh samples that were then analyzed immediately after they were taken, inter-eye differences for Na and Cl were found to be 0 to 9 mmol/L or 0 to 8.8%, respectively (6). In our study, both the refrigerated and frozen and thawed samples maintained correlation with inter-eye differences for Na < 2% and Cl < 3%. In contrast to the study of Pounder et al. which showed inter-eye differences for K of 0 to 21.8%, our study indicated inter-eye differences for K at <4% for both refrigerated and frozen and thawed samples. In addition, we noted no significant differences between eyes for glucose and urea nitrogen measurements with differences of <8% and <2%, respectively. Differences were found, however, between measured and calculated osmolalities of frozen specimens. (Refrigerated specimens were not tested). The mean calculated osmolality was significantly lower than the measured osmolality. The calculated value, mean 305 mOsmol/kg, was much more consistent with normal serum osmolality than the measured value, mean 439 mOsoml/kg. Sturner and co-workers have previously shown the measured osmolality of VH ranged for 280 to 350 mOsml/kg; with proportional increases when ethanol was present (12). In cases negative for ethanol, a reference range of 288 to 323 mOsmol/kg was found. We currently have not identified the mechanism or analytes responsible for the large difference found between measured and calculated values.

We also investigated the potential of measuring keto-acids in VH for detecting ketoacidosis in deaths with unremarkable or minimal autopsy findings. Sudden deaths in chronic alcoholics frequently show this pattern. Laboratory measurement of blood and VH acetone and acetoacetate are commonly accepted methods of detecting ketoacidosis. The ratio of beta-hydroxybutyrate to acetoacetate in alcoholic ketoacidosis, has been shown to be as high as 7.2:1 (13,14). Hence measurement of acetone or acetoacetate may give a falsely negative or inconclusive result, potentially obscuring the correct clinical picture. Our study provides evidence that beta-hydroxybutyrate is detectable in VH using a rapid point-of-care method, shows no significant inter-eye differences, and appears to be more sensitive than either acetone or acetoacetate in detecting increased keto-acids, based on a reference limit (0.6 mmol/L) established in clinical studies using healthy volunteers (15). We suggest that larger studies be performed to better establish postmortem beta-hydroxybutyrate concentrations, with a clinical cause of death correlation. A limitation of the study was that beta-hydroxybutyrate results were only performed in singlet. Therefore, we were unable to determine whether some of the cases (Nos. 1, 11) that showed large between eye differences were due to method imprecision or were true differences. However, both cases showed increased concentrations for beta-hydroxybutyrate in both eyes. Additional studies are necessary to establish the specificity of beta-hydroxybutyrate.

Conclusion

We have shown the reproducibility of analyte measurements in VH samples stored refrigerated or frozen for up to one year. Importantly, we demonstrated small but significant increases in sodium,

potassium, and chloride after refrigeration at 4°C for 6–12 months. Thus, we recommend specimens be stored frozen. We also confirm the correlation of electrolytes, glucose, and urea nitrogen concentrations between eyes. Our data confirm the reliability of the Hitachi system for VH analyte determinations, without differences between eyes. Further studies are needed to understand the cause or causes of differences between calculated and measured osmolalities. Finally, our preliminary observations are encouraging for the testing of beta-hydroxybutyrate in place of or as a supplement to acetone and acetoacetate to assist in the detection of keto-acids and in determining the cause death.

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