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TECHNICAL NOTE

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Hyaluronidase as a Liquefying Agent for Chemical Analysis of Vitreous Fluid*

ABSTRACT: Vitreous humor is a suitable specimen for postmortem clinical chemistry because the analytes remain relatively stable after death and they closely reflect blood levels immediately prior to death. The viscous nature of vitreous fluid, however, presents analytical problems including imprecision and inaccuracy. Various preanalytical treatments, such as boiling, high speed centrifugation, microfiltration and dilution have been used. These techniques are labor intensive and add to imprecision and inaccuracy. Because glycosaminoglycans contribute significantly to the viscosity of vitreous humor, we used hyaluronidase as a liquefying agent. We compared the results of analyses in 33 vitreous humor specimens after hyaluronidase treatment with the results after either no treatment or specimen dilution. Seventeen of the 33 specimens could not be analyzed without dilution. Even after dilution, several analytes still could not be measured. Hyaluronidase treatment negated the need for sample dilution and had no significant effect on the analyses.

KEYWORDS: forensic science, hyaluronidase, vitreous humor, postmortem chemistry

Vitreous humor is a preferred specimen for postmortem clinical chemistry analysis because, unlike blood, its chemical composition changes slowly after death. Several analyte concentrations in vitreous humor reflect blood levels immediately prior to death and are frequently used for the postmortem diagnosis of various pathological conditions (1). Glucose and acetone can aid in the postmortem diagnosis of diabetes (1). Potassium is used by some for the estimation of postmortem interval (2,3). Sodium and chloride levels can disclose evidence of severe dehydration (4) or water intoxication (5). Urea and creatinine can disclose evidence of renal failure (1).

The chemical analysis of vitreous humor presents special challenges. Problems with accuracy and reproducibility have been reported, including unexplained differences in results from the left and right eye (6,7), unexplained variations in results from different instruments (8) and unexplained imprecision (9). Also laboratories commonly report "sample not suitable for analysis" when a chemistry analyzer cannot pipette a vitreous fluid sample. Various preanalytical treatments, such as boiling, high speed centrifugation, microfiltration, and dilution are labor intensive and add to imprecision and inaccuracy (8,9). These variations can pose real problems, potentially leading to misinterpretations of the results.

Most of these problems in chemical analysis are due to the very viscous nature of vitreous humor. Glycosaminoglycans, collagen, and the interactions between these are responsible for the high viscosity of vitreous humor. Because hyaluronic acid is the predominant glycosaminoglycan in the human vitreous fluid (10), we used hyaluronidase as a liquefying agent.

Materials and Methods

Vitreous humor specimens were obtained from the Office of the Jackson County Medical Examiner in Kansas City, MO. A 18 gauze needle was inserted into one or more eyes globes. The vitreous humor was gently withdrawn by syringe and placed in a red top vacutainer tube. The specimen were stored in a refrigerator. The analyses were completed with 12–48 h of sample collection. Hyaluronidase type 1 (catalog number H3506) was obtained from Sigma Chemical Company in St. Louis, MO. We analyzed aliquots of a specimen both with and without hyaluronidase treatment. We first tried to analyze the untreated aliquot without dilution, but we diluted the aliquot with water to reduce viscosity if we could not obtain results. We treated the other aliquot with few crystals of hyaluronidase to a concentration of 1–2 mg/mL. As described in the results and discussion section, even very high concentrations of hyaluronidase does not effect any of the analytes studied; a laboratory can standardize its own convenience way of treating samples such as using small spatula or wooden stick or preparing concentrated solution of hyaluronidase and treating sample with a fixed volume. The treated aliquots were left at room temperature for 5 min before analysis.

The samples were analyzed on a Vitros Chemistry System (Ortho Diagnostics, Rochester, NY), an autoanalyzer. The system uses dry-slide technology with following methods and principles. All analytes are measured directly without predilution.

Glucose—10 μL of sample is deposited on a slide containing glucose oxidase, peroxidase, 4-aminoantipyrine and 1,7-dihydroxynaphthalene. Hydrogen peroxide generated by the action of glucose oxidase on glucose reacts with 4-aminoantipyrine

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and 1,7-dihydroxynaphthalene to form red color. The intensity of the color is measured by reflectance at 540 nm and is proportional to the glucose concentration.

Sodium, Potassium and Chloride—These ions are measured by ion-selective electrodes potentiometry using dry-multilayered slides. The slide consists of two ion-selective electrodes, one for sample and the other for reference fluid. 10 μL of sample and reference fluid are deposited on the slide. The potential difference poised between the two electrodes is proportional to the concentration of the analyte.

Urea—10 μL of sample is deposited on a slide containing urease and ammonia indicator. Urea in a sample reacts with urease to form ammonia which reacts with indicator dye to form a colored complex. The colored complex is measured by reflectance at 670 nm. The instrument provides urea results as blood urea nitrogen (BUN).

Creatinine—10 μL of sample is deposited on a slide where the following reactions take place to form a colored complex. The names of the enzymes catalyzing each reaction are given in brackets. The intensity of the colored complex is measured by reflectance at 670 nm and is proportional to the concentration of creatinine in the sample.

Creatinine + H_2O (creatinine amidohydrolase) Creatine

Creatine + H_2O (creatine amidinohydrolase) Sarcosine + Urea

Sarcosine + O_2 + H_2O (sarcosine oxidase) Glycine + Formaldehyde + H_2O_2

H_2O_2 + Leuco Dye (Peroxidase) Colored Complex

The stability of the calibration curves was evaluated by analyzing human serum controls (Biorad Laboratories, Irvine, CA) with each run. Also, the controls were used to look at the effect of hyaluronidase on various analytes.

To rule out that some of these problems are not instrument related and hyaluronidase method works on other instruments, glucose, potassium, sodium and chloride were also measured on Radiometer ABL735 analyzer (Copenhagen, Denmark). Creatinine was also measured on Roche Cobas Mira (Indianapolis, IN) using alkaline picrate method.

Results and Discussion

The analysis of several chemistry analytes in vitreous humor is important in a death investigation. However, this analysis frequently can be impaired by high viscosity of vitreous humor. In recent years, this problem seems to have been compounded by clot detection systems in chemistry analyzers. Most of the chemistry analyzers now detect viscous fluid as clot and will not perform the analysis.

Various techniques to reduce viscosity have been tried. Centrifugation and filtration of vitreous fluid may eliminate certain components and can lead to inaccurate results. Recently, heating

TABLE 1—*Effect of hyaluronidase on chemistry analytes. Asterisks (∗) in the table indicate that the analyte(s) could not be measured. Hyphens (-) represent the specimen quantity was not sufficient. All hyaluronidase treated samples were analyzed without dilution.*

		Glucose (mg/dL)		Potassium (mmol/L)		Sodium (mmol/L)		Chloride (mmol/L)		BUN (mg/dL)		Creatinine (mg/dL)	
Sample	Dilution	−HL	+HL	−HL	+HL	−HL	+HL	−HL	+HL	−HL	+HL	−HL	+HL
1	None	25	32	11	11	139	141	125	128	8	8	0.4	0.3
2	None	<20	<20	∗	∗	131	139	117	122	7	7	-	-
3	None	55	56	9	10	134	142	115	120	-	18	1.0	0.8
4	None	291	304	-	-	113	117	87	90	53	53	∗	∗
5	None	∗	648	∗	8	173	173	141	146	76	67	1.9	1.4
6	None	<20	<20	-	-	138	138	123	124	9	9	0.8	0.8
7	None	<20	<20	10	11	135	135	121	129	7	7	0.2	-
8	None	<20	<20	12	12	130	134	117	-	7	-	0.3	0.3
9	None	<20	<20	9	9	∗	130	113	117	20	17	∗	0.9
10	None	10	10	7	7	148	147	131	129	31	32	0.9	0.8
11	None	16	15	7	7	134	140	121	126	5	5	0.4	0.4
12	None	483	500	10	10	136	141	120	124	18	18	0.8	0.8
13	None	<10	<10	14	15	131	137	117	121	8	8	0.6	0.6
14	None	10	13	8	8	132	129	114	119	10	11	1.1	1.0
15	None	<10	<10	9	10	137	142	122	127	4	4	0.6	0.5
16	None	38	37	7	7	139	144	123	127	16	15	0.6	0.5
17	1:2	12	12	9	9	138	143	123	126	36	34	3.4	2.8
18	1:2	24	24	6	6	135	133	116	119	13	12	0.6	0.5
19	1:2	65	60	10	10	142	140	124	124	20	21	0.4	0.3
20	1:2	∗	15	7	7	∗	144	125	130	10	12	0.5	0.4
21	1:2	∗	-	9	9	∗	138	∗	120	21	20	1.1	1.0
22	1:2	<20	<20	7	7	∗	145	∗	126	∗	17	∗	0.6
23	1:2	<20	<20	-	-	-	129	-	113	-	14	∗	0.6
24	1:2	∗	291	∗	9	∗	158	∗	135	∗	28	∗	1.0
25	1:3	<20	<20	10	9	∗	142	∗	117	8	10	0.4	0.3
26	1:3	<20	<20	10	10	∗	148	∗	129	12	9	0.9	0.6
27	1:3	<20	<20	9	9	∗	141	∗	127	∗	15	0.4	0.6
28	1:3	<10	<10	11	10	∗	143	∗	129	6	7	0.7	0.6
29	1:4	48	37	8	7	∗	156	∗	138	6	6	0.6	0.3
30	1:4	94	91	14	14	-	149	-	116	14	14	1.1	1.3
31	1:6	173	134	14	12	∗	142	∗	124	36	36	1.2	0.6
32	1:6	<20	<20	7	7	137	135	119	115	19	17	1.1	0.7
33	1:6	<20	<20	-	-	∗	137	130	123	12	12	0.9	-

−HL and +HL represent without and with hyaluronidase treatment respectively.

TABLE 2—*Effect of different concentrations of hyaluronidase on chemistry analytes in human serum controls. Two different concentrations of controls were tested.*

Sample	Glucose (mg/dL)	Potassium (mmol/L)	Sodium (mmol/L)	Chloride (mmol/L)	BUN (mg/dL)	Creatinine (mg/dL)
Control 1, untreated	99	4.0	154	100	14	0.5
Control 1, HL 1 mg/mL	98	4.0	154	100	14	0.5
Control 1, HL 20 mg/mL	99	4.0	154	100	14	0.5
Control 2, untreated	285	6.2	132	88	43	6.1
Control 2, HL 1 mg/mL	283	6.1	132	88	42	6.0
Control 2, HL 20 mg/mL	283	6.1	132	88	43	6.0

TABLE 3—*Effect of different concentrations of hyaluronidase (HL) on chemistry analytes in vitreous samples. Two different concentrations were tested.*

Sample	Glucose (mg/dL)	Potassium (mmol/L)	Sodium (mmol/L)	Chloride (mmol/L)	BUN (mg/dL)	Creatinine (mg/dL)
Vitreous 1, untreated	29	6.0	135	116	24	1.5
Vitreous 1, HL 1 mg/mL	30	6.1	134	114	24	1.4
Vitreous 1, HL 20 mg/mL	31	6.1	136	116	25	1.4
Vitreous 2, untreated	106	9.6	163	136	27	3.3
Vitreous 2, HL 1 mg/mL	111	9.3	161	143	23	2.8
Vitreous 2, HL 20 mg/mL	113	9.3	161	142	23	2.9

the vitreous fluid to reduce the viscosity has been proposed (9). Although heating did not affect sodium, potassium, chloride, urea and creatinine levels, the technique is laborious and renders the sample unsuitable for volatile analysis.

Mucopolysaccrides in vitreous fluid are responsible for its viscosity. After treatment with hyaluronidase, a reduction in viscosity eliminated pipetting and clot detection errors. Table 1 shows the effect of hyaluronidase treatment on the measurement of glucose, potassium, sodium, chloride, urea and creatinine. Analysis of 17 out of 33 (51%) untreated, undiluted samples could not be performed due to pipetting problems or sample not suitable for analysis by the instrument. When the analysis could not be performed on an untreated, undiluted sample, the sample was diluted and reanalyzed. Despite dilution, 9%, 3%, 33%, 27%, 9% and 9% samples for glucose, potassium, sodium, chloride, BUN and creatinine respectively could not me measured. Whenever the values were obtained, on undiluted, untreated or diluted samples, no clinical significant differences were noted in any of the analytes measured after hyaluronidase treatment (Table 1). No clinical differences for glucose, potassium, sodium, chloride, BUN and creatinine were defined as average differences less than 6 or 10%, 0.5, 4, 5%, 2 or 9% and 0.3 or 15% respectively. These limits are from Clinical Laboratory Improvement Act (CLIA) and are widely used to grade clinical laboratories for proficiency testing (11) and seem appropriate to evaluate clinical insignificance between the two measurements i.e., before and after hyaluronidase treatment. To further rule out the effect of hyaluronidase on these analytes, hyaluronidase was added to two controls. Concentrations even 10–20 times higher than used in the study had no effect on any of the analytes (Table 2). Similar finding were noted when vitreous fluid was used (Table 3).

To investigate the hyaluronidase method, three samples were compared with a previous published pretreatment method in which vitreous samples are heated at 100°C (9). No difference was noted, in any of the analytes studied, between hyaluronidase treatment and heating samples at 100°C for 5 min (Table 4). To rule out that some of these problems are not instrument related and hyaluronidase method works with other methods, these samples were run on other instruments. No significant difference was noted between the instruments (Table 4).

TABLE 4—*Comparison of hyaluronidase (HL) method with pretreatment heating method (100°C for 5 min) and other instruments.*

	Sample #	HL	Heating	2nd Method
Glucose (mg/dL)	1	11	12	11
	2	25	25	28
	3	13	12	11
Potassium (mmol/L)	1	9.7	9.4	9.6
	2	7.9	7.8	7.9
	3	8.0	8.0	8.1
Sodium (mmol/L)	1	168	162	165
	2	145	144	147
	3	144	143	144
Chloride (mmol/L)	1	148	147	148
	2	126	129	127
	3	128	129	127
BUN (mg/dL)	1	60	63	ND
	2	7	7	ND
	3	13	13	ND
Creatinine (mg/dL)	1	1.2	1.4	1.3
	2	0.5	0.7	0.6
	3	0.7	0.8	0.8

NOTE: Second instrument for glucose, potassium, sodium and chloride measurement was Radiometer ABL 735 analyzer (Copenhagen, Denmark). Second instrument for creatinine was Roche Cobas Mira (Indianapolis, IN). Due to unavailability of an alternate method, BUN was not determined (ND) by another method.

In conclusion, a simple hyaluronidase treatment can liquefy vitreous humor. The samples are then easy to process and give reliable results. Also, hyaluronidase treatment negates the need for sample manipulations like dilution, centrifugation, filtration or heating which can lead to imprecision and inaccuracy.

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