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Variations in vitreous humor chemical values as a result of pre-analytical treatment

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ABSTRACT

Vitreous humor (VH) is used for postmortem diagnosis of metabolic diseases and to clarify the postmortem interval. Because of its viscous nature, this fluid has to be liquefied prior to analysis; however variations in measured concentrations of the analytes are ascribed to different pre-analytical treatment methods with regard to. The aim of this study was to compare different pre-analytical methods. Centrifugation, heat treatment, enzymatic digestion and liquefying by ultrasound were compared using a collection of 120 samples obtained from 2003 to 2007. The determined parameters were: sodium, potassium, chloride, calcium, glucose, creatinine, urea and lactate. Analyses were performed either photometrically or by using ion-selective electrodes.

Heat and hyaluronidase treatment generate slightly higher and lower values in the measurement of electrolytes and glucose. However, in the determination of calcium concentration, both methods (heat especially) are associated with extreme low or high values. Only differences between ultrasound and centrifugation treatment show comparatively small variations and are close to instrument accuracy. Therefore, we recommend centrifugation, combined with mixing, as the best and easiest method in which to prepare frozen samples for analysis. Additionally, the measurement of lactate shows that analytical methods, calibrated for serum and urine, cannot be easily applied for VH.

1. Introduction

Vitreous humor (VH), compared to cerebral spinal fluid (CSF) and blood, is the most commonly analysed fluid compartment for biochemical investigations, due to delayed autolytic changes, and is easily obtainable without contamination [1,3,10]. VH chemistry is used for determination of time since death [8,9,12] – even in late postmortem intervals [7] – as well as for postmortem diagnosis of metabolic diseases (e.g., diabetes mellitus, electrolyte imbalances, urea retention) [4,11,15]. However, one of the main handicaps in VH analysis is that analytical methods, currently applied, are calibrated and validated for serum or urine analysis [13]. Furthermore, variations in VH biochemical values, due to instrumentation, have been already observed some decades ago [2]. Another problem, due to the high viscosity of VH, is the intra-individual difference, as seen in the differences in measurements of the same electrolyte using the same VH and the same analyzer [14]. Therefore, several attempts have been made to reduce viscosity by different methods of sample pre-treatment. To reduce VH viscosity, the use of hyaluronidase (HY.) [6], heating (He.) [14], liquefying by ultrasonification (U.) [16], and centrifugation (C.) [8], as single or combined methods, have been recommended. This present study compares these 4 methods of pre-analytical treatment. Since all specimen were centrifuged twice prior to analysis, centrifugation was really compared with centrifugation combined with hyaluronidase, heating, and ultrasonification.

2. Materials and methods

2.1. Experimental setup and sample pre-treatment

Between 2003 and 2007 VH was obtained during 120 routine medico-legal autopsies using 20-gauge needles. In each case, VH – as much as was possible – was gently withdrawn by syringe. The sample of each eye was separately stored frozen at −18 °C in 13-ml sample tubes (polypropylene container, screw top) to prevent degradation.

All specimens, with a total volume of at least 2 ml, were analysed. After thawing at room temperature each sample was treated separately. All samples were vortexed for 30 s using the highest level. Subsequently, all samples were centrifuged (8 min, 1650 × *g*) and only the supernatant was used for analysis. The supernatant was divided into four aliquots. Each aliquot was approximately 0.5 ml, and VH was aspirated from the base of the specimen. Each aliquot passed through one of the 4 different methods of sample pre-treatment, in randomised sequence:

1. For enzymatic digestion, hyaluronidase from bovine testes, from Sigma Chemical Company in St. Louis, MO (catalogue number H3506), was used. The aliquot was treated with a few crystals of hyaluronidase, resulting in a concentration of 1–

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Table 1
Reaction type, type of measurement and wavelength of each analyte.

Analyte	Reaction type	Type of measurement	Wavelength
Calcium	o-Cresolphtahalein-complexon-rection	Trichromatic endpoint technique	577, 540
Creatinine	Alkaline Picrate method	Trichromatic rate technique	510,600
Urea	Urease-GLDH-method	Bichromatic rate technique	340, 383
Glucose	Hexokinase-method	Trichromatic endpoint technique	340, 383
Lactate	Lactate + $NAD^+ \geq$ pyruvate + NADH + H	Two-filter endpoint technique	340–383

2 mg/ml. In order to achieve good mixture, samples were vortexed at the highest level.
2. For heat liquefaction, VH was filled in glass tubes closed by a screw top. The glass tube was heated in a heating block for approximately 5 min at 100 °C. Then the specimen was cooled at room temperature for 30 min and centrifuged at 1650 × *g* for 5 min. Only the supernatant was used for analysis.
3. For salvation by ultrasound, the aliquot was treated in an ultrasonic bath for 15 min at 20 °C.
4. When vitreous humor had to be solvated by centrifugation, no further treatment was necessary.

Subsequently, all specimen were directly stored frozen at −25 °C. After a second thawing, the specimens were centrifuged at 13,100 × *g* for 10 min prior to analysis.

2.2. Chemicals and instrumentation

Biochemical analysis was performed on Dimension® RxL Max® Integrated Chemistry System (Siemens Healthcare Diagnostics Inc., Newark DE 19714, U.S.A.), which is an autoanalyzer with integrated Multisensor Technology (IMT) called QuickLYTE®. Tests had been performed by routine analysis at the Institute of Clinical Chemistry and Pharmacology, University of Bonn. The analytes were measured with the following methods and principles.

Sodium (Na), potassium (K), chloride (Cl): these ions were measured by an ion-selective electrode with indirect potentiometry. An electrical potential was developed in the IMT that is proportional to the activity of each specific ion in the sample. The samples were not diluted prior to analysis. To obtain results within the measurement range for potassium, the samples were treated like urine samples. Therefore, measurement ranges of the analytes were: Na 50–200 mmol/l; K 1–300 mmol/l (established in urine); and Cl 50–200 mmol/l.

Calcium (Ca), glucose (Glu), lactate (Lac), urea (Urea), creatinine (Crea): these parameters were determined photometrically. The specifications are as follows: wavelength range; automatic wavelength selection by 10 position filter wheel (340, 383, 405, 452, 510, 540, 577, 600, 700 and 293 nm); and photometric range: −2.5 to 2.5 mAU for 293 nm and −1.5 to 1.5 mAU for other wavelengths). Table 1 shows the reaction type, the wavelength and the type of measurement for each analyte. Measurement ranges for the photometrically determined parameters for serum were: Gluc 0–500 mg/dl; Lac 0.3–15 mmol/l; Urea 0–150 mg/dl; and Crea 0–20 mg/dl.

To obtain results within the measurement range for calcium, samples were treated like urine samples. Therefore, the measurement range for Ca was 0.0–15.0 mmol/l. The concentration of Lac was determined in diluted samples (diluted with NaCl 1:6) as soon as the measured concentrations were higher than 15 mmol/l. Therefore, this was the last parameter to be measured.

2.3. Statistical analysis

The 4 methods of pre-analytical treatment were compared, pair-wise, resulting in the following six combinations:

1) Heat–Ultrasound (He.–U.).
2) Heat–Centrifugation (He.–C.).
3) Heat–Hyaluronidase (He.–Hy.).
4) Hyaluronidase–Ultrasound (Hy.–U.).

Table 2
Descriptive statistics.

Analyte	Mode of pre-treatment	*n*	Min	Mean	Max	SD	Median
Na [mmol/1]	Heat	120	99	136.35	165	9.799	136
	Hyaluronidase	120	97	135.23	164	9.767	135
	Ultrasound	120	98	135.57	166	9.864	136
	Centrifugation	120	97	135.47	163	9.786	135
K [mmol/1]	Heat	120	6	16.54	27	4.856	16.38
	Hyaluronidase	120	6	16.38	27	4.76	16.3
	Ultrasound	120	7	16.44	27	4.811	16.41
	Centrifugation	120	6	16.45	26	4.813	16.49
Cl [mmol/1]	Heat	120	92	125.76	151	9.229	126
	Hyaluronidase	120	91	124.53	150	9.161	125.5
	Ultrasound	120	91	124.85	151	9.347	126
	Centrifugation	120	90	125.01	149	9.329	125.5
Ca [mmol/1]	Heat	120	0.23	0.8263	2.11	0.45611	0.74
	Hyaluronidase	120	0.23	0.9398	2.31	0.50622	0.885
	Ultrasound	120	0.2	0.9115	2.31	0.48769	0.875
	Centrifugation	120	0.23	0.9153	2.31	0.49037	0.865
Creatinine [mg/dl]	Heat	120	0	0.8582	4.17	0.69398	0.705
	Hyaluronidase	120	0	0.853	4.05	0.67946	0.7
	Ultrasound	120	0	0.8301	3.93	0.67257	0.68
	Centrifugation	120	0	0.8361	3.96	0.67326	0.7
Urea [mg/dl]	Heat	120	14	62.04	236	41.729	48
	Hyaluronidase	120	15	61.72	227	41.201	47
	Ultrasound	120	15	61.93	236	41.665	47
	Centrifugation	120	14	62.18	231	41.981	48
Glucose [mg/dl]	Heat	119	0	7.95	132	21.02	0
	Hyaluronidase	119	0	8.37	133	20.83	1
	Ultrasound	119	0	8.14	132	20.67	1
	Centrifugation	119	0	8.24	134	20.87	1
Lactate [mmol/1]	Heat	103	0	26.088	51.1	8.93	25.90
	Hyaluronidase	103	0	25.709	42.7	8.35	25.70
	Ultrasound	103	0	25.756	48.2	8.65	25.70