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Stability of endogenous GHB in vitreous humor *vs* peripheral blood in dead bodies

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ABSTRACT

For the first time, the stability of GHB was tested in post-mortem peripheral blood and vitreous humor samples, collected from 22 dead bodies at two different times: at the external body examination at the place of death and then during autopsy.

An *ad hoc* method for the detection and quantification of GHB in vitreous humor by gas chromatography coupled to mass spectrometry (GC–MS) was developed and validated, with a good linearity between 0.1 and 50 μg/mL ($r^2 = 0.991$) and a precision and accuracy always better than 10% and an analytical recovery higher than 90%.

The geometric mean of GHB concentration in the 22 peripheral blood samples at t_0 was: 3.6 μg/mL (95% CI: 2.3–5.9 μg/mL) and at t_1 it was 7.4 μg/mL (95% CI: 5.0–10.9 μg/mL); that of GHB in the 22 vitreous humor at t_0 was: 2.5 μg/mL (95% CI: 1.5–4.1 μg/mL) and at t_1 it was 3.0 μg/mL (95% CI: 1.9–4.8 μg/mL). There was no significant difference between the GHB concentrations in vitreous humor and peripheral blood at t_0 in all the samples ($p > 0.10$). Conversely at t_1, the increase of GHB in the peripheral blood was significantly increased by a 102% (range: 86–120%) ($p < 0.001$ *vs* t_0), while in the vitreous humor only a slight increase by 19% was observed (range: 16–21%) ($p > 0.05$ *vs* t_0). Finally at t_1, GHB values in the two matrices were statistically different, being that of peripheral blood higher ($p < 0.01$).

This study demonstrated the usefulness of vitreous humor as a more stable alternative matrix in comparison to peripheral blood for the post-mortem determination of endogenous GHB.

1. Introduction

Gamma-hydroxybutiric acid (GHB) is an endogenous precursor and a metabolite of the chief inhibitory neurotransmitter gamma-aminobutyric acid (GABA) and an exogenous xenobiotic used as medicament for narcolepsy and alcohol withdrawal and as a recreational club drug [1]. Taking into account the dual nature of this compound, endogenous and exogenous, interpretative problems can frequently arise when discriminating between endogenous production and exogenous exposure [2]. Therefore, accurate concentration cut-offs are necessary in toxicological investigations. The interpretation of GHB values is even more complicated when dealing with post mortem samples, because of the high endogenous concentrations detectable in samples collected from dead bodies [1,2]. The endogenous concentrations of GHB are higher in blood samples collected during autopsies compared with specimens from living people [1]. Because of the wide variation in endogenous GHB concentrations in post-mortem blood cut-off concentrations higher than the ones used in ante-mortem samples, are needed to differentiate ante-mortem ingestion from post-mortem production of GHB. Toxic and even lethal doses of GHB have been detected in post-mortem blood samples, even in non GHB-related deaths. Cut off value of 30 μg/mL has been proposed for whole blood, as far as there are no signs of advanced body putrefaction. Moreover, 50 μg/mL has been suggested as cut-off value to discriminate GHB endogenous production from active consumption [3].

In post-mortem settings, GHB has been measured not only in conventional matrices such as blood and urine [2,4–8], but also in

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alternative matrices such as hair [9,10]. For other forensic matrices, like vitreous humor, only few studies are currently available [2,8,11].

As already reported by other authors [1,8], vitreous humor is a relatively isolated matrix, located within the globe of the eye between the retina and the lens, which resists putrefaction longer than other body fluids, such as blood. For this reason, it may represent a promising matrix for the determination of the actual concentration of GHB in deceased persons. In forensic toxicology, vitreous humor has served as an alternative matrix for more than 50 years. Its lack of vascularization, anatomic remoteness from viscera, and relative protection by the eyeball render make it a useful alternative when blood cannot be sampled (exsanguinated or fragmentary cadaver) or in the case of suspected postmortem redistribution or contamination by bacteria or chemicals (*e.g.*, embalming). As it is easy to sample, and because it can be used for immunological analysis of certain groups of chemical substances, vitreous humor has even been recommended for immunoenzymatic screening on the site where a victim was discovered [12]. Vitreous humor GHB concentration of 58 μg/mL was found in a case of drug facilitated sexual assault, in which a six-year-old girl died following sedation with GHB. Her death was attributed to a lethal GHB intoxication [13].

The objectives of this research was to determine the stability of GHB in post-mortem peripheral blood and vitreous humor samples, collected from 22 deceased persons at two different times: at the external body examination at the place of death and then during autopsy. An *ad hoc* method for the detection and quantification of GHB in vitreous humor by gas chromatography coupled to mass spectrometry (GC–MS) was developed and validated, whereas for the analysis of GHB in blood samples a previously published method was used.

2. Materials and methods

2.1. Reagents

GHB and GHB-d_6, used as internal standard, were acquired from Cerilliant-Sigma-Aldrich (St. Louis, MO, USA). Ethyl-acetate was obtained from Carlo Erba® (Milan, Italy). Deionized water was purified using a Millipore-Elix® 70 system from Merck Millipore. N,O bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) derivatizing agent was purchased from Supelco (Bellefonte, USA) and a 0.9% saline (NaCl) was purchased from Fresenius Kabi Italia (Verona, Italy).

2.2. Samples collection

The selection criteria for cases inclusion to the study were: (1) non-GHB related fatalities and (2) availability of both blood and vitreous humor specimens at the two time points, as above described. Twenty-two post-mortem cases were selected (14 males and 8 females, mean age 40.9 ± 18.34 years) and two peripheral blood and two vitreous humor samples (0.5–1 mL each) were collected from each case (using always the same eye for the same case) during the external body examination at the place of death (t_0), which was performed in all cases within 12 h from death (mean PMI and standard deviation: 4.54 ± 2.28 h) and during autopsy (t_1), which was performed in all cases between 25 and 144 h (mean PMI and standard deviation: 50.6 ± 32.6 h). Age, sex and cause of death and raw data at t_0 and t_1 of each of the cases selected are reported in Table I. All the cases included in this study underwent a general blood screening for drugs of abuse by liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [14], which always resulted negative. All bodies were kept at room temperature for the first 24 h and then refrigerated at 4 °C until autopsy. Once collected, peripheral blood and vitreous humor specimens were immediately stored at −20 °C until analysis, which is the optimal storage temperature as described elsewhere [5]. No preservatives were used.

The analysis of vitreous humor samples was performed according to the method below reported, whereas peripheral blood samples were analyzed by GC–MS after liquid liquid extraction (LLE) according to a previously published method [15].

2.3. Vitreous humor extraction

To 100 μL sample (calibrators and controls), 1 mL deionized water, 5 μL GHB-d_6 (100 ng/μL), 1 mL of sodium acetate buffer 1 M (pH 5) and 4 mL of ethyl acetate were added. The samples were mechanically mixed (vortex) for 1 min and then centrifuged for

Table I
Sex, age, cause of death, GHB concentration in vitreous humor and peripheral blood of the 22 study cases at the external body examination at the place of death (t_0) and then during autopsy (t_1).

				GHB concentration (μg/mL)			
Cases	Sex	Age	Cause of death	t_0 vitreous humor	t_1 vitreous humor	t_0 peripheral blood	t_1 peripheral blood
1	M	24	Hanging	3.0	3.3	12.1	19.2
2	M	62	Myocardial infarction	3.7	4.1	5.4	8.1
3	M	76	Pleural mesothelioma	0.6	0.7	0.7	1.4
4	M	23	Firearm injuries	0.2	0.3	0.8	1.8
5	F	46	Pulmonary thromboembolism	1.2	1.9	2.1	3.9
6	M	48	Myocardial infarction	6.9	7.2	8.3	12.9
7	M	60	Cardiac tamponade	5.1	5.5	6.0	6.2
8	M	34	Firearm injuries	3.5	4.2	5.2	9.1
9	M	67	Pulmonary thromboembolism	9.1	12.5	19.1	34.1
10	M	48	Myocardial infarction	1.0	1.1	1.3	2.3
11	F	69	Pulmonary thromboembolism	2.5	2.9	3.1	5.8
12	M	26	Firearm injuries	8.6	10.0	9.3	14.2
13	F	19	Blunt chest trauma	0.7	0.8	0.8	4.5
14	F	21	Hypereosinophilic syndrome	7.4	8.0	32.1	53.5
15	F	36	Ligature strangulation	1.5	1.6	2.2	4.3
16	M	25	Abdominal trauma	15.1	16.0	11.3	16.5
17	F	42	Intracerebral haemorrhage	2.3	3.0	1.9	7.3
18	F	17	Congenital coronary artery anomaly	1.8	2.1	2.1	6.8
19	M	55	Firearm injuries	4.1	4.5	5.6	10.0
20	M	28	Chest and abdominal stab wounds	12.2	14.0	5.9	9.4
21	F	22	Thrombotic thrombocytopenic purpura	0.7	0.9	1.2	6.2
22	M	51	Myocardial infarction	1.2	1.5	1.5	3.3