Original article

A fast and reliable method for GHB quantitation in whole blood by GC–MS/MS (TQD) for forensic purposes

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ABSTRACT

Gamma-Hydroxybutyric Acid (GHB) is an endogenous compound with a story of clinical use since the 1960's. However, due to its secondary effects, it has become a controlled substance, entering the illicit market.

A fully validated, sensitive and reproducible method for the quantification of GHB by methanolic precipitation and GC-MS/MS (TQD) in whole blood is presented. Using 100 μL of whole blood, obtained results included a LOD and LLOQ of 0.1 mg/L and a recovery of 86% in a working range between 0.1 and 100 mg/L.

This method is sensitive and specific to detect the presence of GHB in small amounts of whole blood (both *ante-mortem* or *post-mortem*), and is, to the authors' knowledge, the first GC-MS-MS TQD method that uses different precursor ions and product ions for the identification of GHB and GHB-D_6 (internal standard). Hence, this method may be especially useful for the study of endogenous values in this biological sample.

Key-words: Forensic Science, Gamma-Hydroxybutyric Acid (GHB), GC-MS/MS, analytical validation, endogenous values, forensic toxicology

1 - Introduction

Gamma-hydroxybutyric acid (GHB) is known to be an endogenous, naturally occurring, short-chained fatty acid found in mammalian tissues, with wide distribution and further action in several brain areas. Although it was first synthesised in 1960, it was rapidly identified as an endogenous compound. Alongside his history of clinical use for some decades, both in Europe and in the United States, its illicit use includes recreational use, muscle building effects in bodybuilders and drug-facilitated sexual abuse, alone or mixed with other substances, as this can be added clandestinely to beverages, due to its odourless and colourless liquid state. The increasing consumption led the public authorities to worry about its safety and effectiveness for licit use without clinical supervision. As so, even with a therapeutic history, it was banned from public sale in the nineties. However, internet sites continued to sell GHB, under several street names [1,2].

Consequent demand for routine toxicological analysis, as, obviously, increased, and GHB analytical detection in biological samples for forensic purposes became part of routine analysis in many toxicological labs. Nevertheless, GHB levels obtained in casework, both in *ante-mortem* and in *post-mortem* samples, always require a careful interpretation, not only due to its endogenous formation, but also due to *post-mortem* production, linked to autolysis and microbial action phenomena, and its specific speed of metabolism and excretion. Thus, the establishment of cut-off values in biological samples became crucial, in order to distinguish external exposure from endogenous values, as GHB levels detection may range from physiological to pharmacological and even toxic concentrations in blood samples, even when there is no suspicions of GHB use [1,5-12].

2 - Material and Methods

2.1 - Materials, standards and chemicals

Pure Gamma-hydroxybutyric acid (GHB) and deuterated internal standard (GHB-D_6) were purchased from Cerilliant Corporation (Round Rock, TX, USA) at concentrations of 1 mg/mL.

All standards and their dilutions were stored at -20ºC. BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) + 1% TMCS (trimethylchlorosilane), was purchased from Sigma Aldrich (Sintra, Portugal).

Individual 100 mg/L stock solutions for both analytes were prepared, starting from 1 mg/mL solutions in methanol. These standard solutions were used to make calibration curves with the following concentrations: 0.1, 1, 2, 5, 10, 20, 50 and 100 mg/L. Standards were made in methanol to determine the limit of detection (LOD), lower limit of quantification (LLOQ) and calibration curves. The concentration of the internal standard was 10 mg/L. Analytical validation was carried out always considering the endogenous context of GHB.

Methanol was of gradient grade and was purchased from E. Merck (Algés, Portugal).

2.2 - Specimen collection

Whole blood samples were collected according to the routine autopsy procedures from the Forensic Pathology Department of the Portuguese National Institute of Legal Medicine and Forensic Sciences, North Branch.

Whole blood samples were immediately frozen, after addition of NaF, as previously suggested [12], in order to avoid *in vitro* changes as to GHB concentration is concerned.

2.3 - Sample preparation and extraction

Ten microlitres of internal standard solution were added to a 100 µL whole blood aliquote. Two hundred microlitres of methanol were added to the eppendorf-type tube. The sample was vortexed, agitated in a roller mixer for 15 minutes and centrifugated at 5000 RPM, for 30

minutes, after which the methanol was transferred to a clean glass vial. Once the methanol dried completely under a stream of nitrogen, 60 µL of BSTFA + TMCS 1% was added. The derivatization was performed at 65ºC for half an hour, followed by instrumental analysis using GC-MS/MS.

2.4 - GC-MS/MS analysis

GC-MS/MS analyses were conducted on a Bruker GC-450 gas chromatograph coupled to a 300-MS Mass spectrometer (Bruker, Columbia, MD, USA). Analyte separation was achieved with a J&W Capillary Column (30 m–0.25 mm i.d. - 0.25 film thickness) (Agilent, Palo Alto, CA, USA).

Instrument control, data acquisition and processing were achieved with the use of a Bruker MS Workstation Software (Version 7.0).

The GC oven temperature ramp was as follows: 60ºC, 2 min hold, increase to 120ºC at a rate of 10ºC/min, then to 300ºC at 30ºC/min, with a final hold for 5 min at the final temperature. The total run time was 16 minutes. 2 µL of samples were injected in *splitless* mode with a column flow rate of 1.3 mL/min. The injection temperature was 250ºC.

The ion source and interface temperatures were 260ºC and 280ºC, respectively. The MS was operated in SIM/SIM mode. The monitorized ions (m/z) were as follows: two transition ions for GHB (233 m/z => 131 m/z and 233 m/z => 143 m/z), with 143 m/z as the quantitation ion. The transition ion for the internal standard (GHB-D6) was 239 m/z => 149 m/z. Collision energy for precursor ions breaking in Q2 was 10V, with a dwell time, per ion, of 0.1 sec. Collision gas was Argon, at a constant pressure of 2.0 mTorr.

The retention times for the analytes were 9.27 min, for GHB, and 9.24 min, for GHB-D_6.

2.5 - Validation studies

To study the compounds behaviour, GHB and GHB-D_6 standards were injected in SCAN mode, in order to evaluate and choose the better precursor ions. To choose the best product-ions, along with the most effective collision energy in Q2, the standards were injected again in SIM/SCAN mode, for an evaluation of obtained spectra and respective analytical signals and intensities. The collision energy was studied from 5 to 50 V, being 10 V the chosen one, based on the above referred characteristics (better analytical signal and intensities for both the product-ions). Finally, the chosen parameters were tested in SIM/SIM mode, in order to corroborate the described options.

The method specificity was evaluated by adding the analyte and the internal standard to 5 aliquots of non-distilled water, with concentrations of 0.1 mg/L and 1 mg/L for GHB and GHB-D_6, respectively. Contemporaneously, 5 aliquots of water, non-fortified, were also tested.

The LOD was tested with five aliquots fortified with 0.1 mg/L of GHB. The LOQ was tested also with five aliquots fortified with 0.1 mg/L.

Extraction recovery was tested by adding GHB, at two different concentrations, before extraction, and GHB-D_6 after extraction. In another set, both GHB and GHB-D_6 were added after the extraction procedure. Each concentration was tested in triplicate (n=12). After obtaining the GHB/GHB-D_6 areas ratios, recovery was determined by calculating the percentage of the reason between areas ratios before and after extraction.

Linearity was studied with a calibration curve based on ten levels, as most equidistant as possible, between 0.1 and 100 mg/L. After r^2 evaluation, 5 injections of aliquots at the lower and higher limits of the studied work range were obtained. The variance (SD^2) homocedasticity was studied, comparing the tested value (F_{calc}) with the reference value (F_{crit}) from the f-test table (d.l.=4).

As mentioned by Peters *et al.* [13], accuracy can be affected by systematic (bias), as well as random (precision), error components. However, the term is often used to simply describe the systematic error component, i.e., in the sense of bias. Trueness, associated with the term "accuracy", and linked to the sense of "bias", may be defined as "the difference between the expectation of the test results and an accepted reference value" [13], and it is usually expressed as a percent deviation from the accepted reference value. Thus, the method accuracy was calculated measuring the analyte concentration at two different levels. For this purpose, duplets of aliquots at 0.5 and 80 mg/L were processed.

Precision may be defined as "the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions and may be considered at three levels: repeatability, intermediate precision and reproducibility" [13]. Within-run or within-day precision are also often used to describe repeatability. Between-day precision may be used to measure the precision components associated to intermediate precision. Reproducibility is not applicable to this context, as it is usually used to evaluate inter-laboratory results and method behaviour [13].

The method was applied to real samples, obtained according to the protocol applied in the Forensic Pathology Department.

3 - Results and discussion

The SCAN analysis of GHB and GHB-D_6 gave some possibilities in terms of ions choice. And although they were not the most intense ones, 233 m/z and 239 m/z were chosen as precursor ions for GHB and GHB-D_6, respectively, due to less influence of possible interferences. The precursor ion of GHB (m/z 233) and the respective analogue deuterated internal standard GHB-D_6 (m/z 239) corresponds to the demethylated molecular ion [14]. In fact, although the ion 147 was the most intense one in the SCAN mode, it was not chosen

since it is present in both compounds, being also common to some silanes and other chromatographic column interferences. After the precursor-ions cleavage in Q2, the chosen product-ions were two transition ions for GHB (233=>131 and 233=>143 m/z), being the fragment ion at m/z 143 the quantitation ion, and the fragment ion at m/z 149 the internal standard signal (GHB-D_6). These transition ions seemed interesting, as, in SIM/SCAN mode, GHB-D_6 behaviour shown a mass spectrum where fragment ions at m/z 137 and 149 are also the most abundant. The difference is justified by the six deuterium atoms, which are allocated to the carbon chain [Figure 1 a) and b)].

Figure 1 a) Chemical Structure of derivatized GHB b) Chemical structure of derivatized GHB-D_6

During the validation process, the first tested parameter was specificity/selectivity. Five fortified samples, with a concentration of 0.1 mg/L, were tested, along with five blank samples. No false positives or negatives were detected. In fact, the target-compound was perfectly identified, using the two precursor-ions peak areas and their relative intensities in percentage, between the base ion peak and the other transition ion, as suggested by international guidelines [15]. Absolute retention time and internal standard relative retention time were also used to validate a positive identification of GHB. Figure 2 shows the chromatogram of a fortified sample at 0,1 mg/L.

The limit of detection (LOD) was estimated from five samples spiked with GHB (0.1 mg/L), and all samples became positive for GHB, without any false negative. The same samples were quantified for consequent determination of the LLOQ, using a contemporary calibration curve. The obtained results showed a coefficient of variation (CV) of 7.0%, lower than the

198 reference values for GC-MS-MS (20%). These results allowed the estimation of 0.1 mg/L
199 both as LOD and LLOQ.
200 For extraction recovery study purposes, a batch of samples (n=6) was prepared, at two
201 different concentrations (0.5 mg/L and 80 mg/L). The samples were extracted and, after
202 extraction and before derivatization, 1 µg/mL of the internal standard was added to the
203 samples. Contemporarily, another batch of samples (n=6) was also extracted, but both the
204 target-compound and the internal standard were added after extraction and before
205 derivatization.
206 The analytical results were compared, allowing the calculation of the extraction recovery in
207 percentage data. Eighty six percent was the calculated recovery for GHB, based on the values
208 from both concentrations, showing good capacities in terms of this parameter.
209 For linearity study purposes a work range from 0.1 to 100 mg/L was established, in order to
210 include both endogenous and possible exogenous values (consensus cut-off for in vivo
211 samples = 10 mg/L ; post-mortem samples = 30 mg/L). The variances homoscedasticity was
212 first studied, with a sample spiked with 0.1 mg/L and another with 100 mg/L, and re-injected,
213 each one, five times in a row. The obtained variances were compared (Var_1=0.00113;
214 Var_2=7.994), with the application of an F test for variance comparisons, being the F_{calc} equal
215 to var_2/var_1 (7419.142). F_{calc} was superior to F_{crit} (4.026), with a degree of confidence of 95%
216 and 4 degrees of liberty. Thus, the method proved to be heterocedastic for the defined work
217 range, meaning that this behaviour must be considered for calibration curve validation. Peters
218 et al. [13] suggest that, in such cases, the data should be mathematically transformed, or a
219 weighted least squares model should be applied. Usually, the factors 1/x or $1/x^2$, i.e., the
220 inverse of the concentration or the inverse of the squared concentration, respectively,
221 adequately compensate for heteroscedasticity. To choose the better factor, different factors

were studied, in terms of coefficient of correlation (r^2) and sum of residues (%). The results are shown in table S1.

The results evaluation suggests the use of 1/x factor, as it has an $r^2 > 0.99$ and, among those with such an r^2, is the one with the minor sum of residues, in percentage.

The intra-day precision was evaluated at two different concentration levels (0.5 mg/L and 80 mg/L), both with CV's less than 15% (0.5 mg/L: 6.63% ; 80 mg/L: 3.17%). Through inter-day precision analysis, three batches were studied, with the application of an ANOVA test for variance analysis, again for two different concentrations. The obtained results are described in Table 1, where we can see that the F value is smaller than the F critical value, for the chosen number of degrees of liberty. On the other hand, the method proved to be accurate (trueness), with 97.97% for 0.5 mg/L, and 104.05% for 80 mg/L.

All the obtained values were below the acceptance criteria for precision (15% R.S.D) and accuracy (bias within 15% of the accepted reference value) suggested by Peters *et al.* [13], making it acceptable for the method parameters' compliance. The method was, thus, applied to real cases.

Whole blood real samples (n=37) were received through a defined period of time, and included male and female corpses, different medico-legal aetiology, a broaden range as to age is concerned, and various results in terms of toxicological analysis (Table 2).

We could observe that the concentrations achieved in all of the real cases were under the cut-off reference levels suggested by previous studies [14], namely 30 mg/L. Needless to say, the analysis of alternative samples, such as hair, should be considered, to discard a possible influence of GHB in the cause of death, whenever there are values higher than the cut-off references in blood samples. It is also important to be aware that there is a rare genetic disorder called GHB aciduria, which results from a failure on semialdehyde dehydrogenase.

246 The subsequent accumulation of GHB arouses, increasing its values to exogenous-
247 consumption-like levels [7,8,16].
248 For a more robust evaluation, the method was compared with published methods using
249 tandem MS analytical procedures for quantitation of GHB applicable to whole blood samples
250 [8,17,18,19]. The first detail to take into account is the absence of GC-MS-MS triple quad
251 based methods, as the only referred method is based on an Ion-Trap Mass Spectrum Detector
252 [8].
253 In fact, the presented method combines, for the first time, the use of different transition ions
254 (both precursor and product-ions) associated to GHB and the deuterated internal standard,
255 which becomes an important advantage, in terms of positive confirmation parameters, namely
256 specificity. Thus, and in defiance of other GC-MS-MS methods, which use both the same
257 product ion (usually, the 147 m/z, from the precursor ions 233 and 239, respectively for GHB
258 and GHB-D_6), with the identification of GHB and GHB-D_6 based only in the retention time,
259 this method guarantees a further reliable, specific and trustable positive identification.
260 The simple sample preparation, based on protein precipitation with methanol, also gave
261 excellent results, both in terms of specificity and matrix interference. In terms of LOD and
262 LLOQ, it is possible to conclude that this method achieved a better value, when compared
263 with LC-MS-MS procedures, representing a significant improvement in terms of analytical
264 quality. In terms of recovery, it should also be noticed that the obtained percentage is similar
265 to methods already described, all of them superior to 80% [4,17,18,19]. This recovery rate
266 also shows that the use of Methanol may be an interesting alternative, avoiding the use of
267 other extraction solvents, such as Acetonitrile [17] or ethyl acetate [20], much more expensive
268 in terms of laboratory acquisition, or even SPE procedures [17,19], more expensive and time-
269 consuming than this simple procedure.
270

4 - Conclusion

A methanolic precipitation - GC/MS/MS method is described for the detection and quantitation of GHB in whole blood samples. This method is specific, precise, and linear, being also sufficiently sensitive to be used both in *post-mortem* and *ante-mortem* samples, even for endogenous levels. Also, the use of a simple and quick extraction procedure is definitely important for a fast laboratory response. In fact, when considering high throughput laboratory analysis, as simpler as a procedure can be, without compromising reliability, accuracy and confidence in the analytical results, more important becomes the procedure for the laboratory capabilities, in terms of time response.

The analytical validation results have shown significant improvements of the analytical quality compared to former methods, mainly in terms of LLOQ, comparing, as an example, with (UHP)LC-MS-MS procedures.

To our knowledge, this is the first GC-MS-MS Triple Quadrupole method that uses different precursor ions and product ions for the identification of GHB and GHB-D_6. This approach represents a further reliable guarantee in terms of positive identification of GHB, adding the advantages, in terms of relative analytical response, of using the analogue deuterated compound as an internal standard. This fact is also evidenced by the good results in terms of precision, accuracy and linearity, as to quantitation is concerned.

This simple, fast, easy and reproducible method is proper for the determination of GHB in whole blood from forensic cases, with good application to laboratory routine analysis and increasing the laboratory response in terms of results for GHB detection and quantitation.

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Table 1

Inter-day precision data.

	SQ	*D.L.*	*MQ*	*F*	*P value*	*F critical*
Between groups (0.5 mg/L)	22.66	2	11.33	5.454394	0.100172	9.552094
Between groups (80.0 mg/L)	406.54	2	203.27	1.164469	0.422397	9.552094

Table 2

Real Cases.

Case number	[GHB] WB (mg/L)	Cause of Death	Age	Gender	Other substances	PMI interval (days)*
1	5.15	Traumatic lesion	92	M	Tramadol ; Sertraline < 25 ng/mL	4
2	7.52	Mechanical asphyxia	55	M	ethanol: 0.1 g/L	3
3	1.82	Natural death	69	M	----	1
4	4.13	Natural death	61	M	----	2
5	3.58	Traumatic Lesion	47	F	Sertraline: 528 ng/mL; Zolpidem < 100 ng/mL	2
6	6.59	Undetermined	63	M	Ethanol: 0.15 g/L; Lidocaíne; Tiapride 1455 ng/mL; Diazepam 6.54 ng/mL; Oxazepam 27.9 ng/mL	3
7	2.60	Natural death	53	M	ethanol; 1.73 g/L	
8	4.00	Natural death	53	F	Diazepam 1.96 ng/mL; Oxazepam 1.2 ng/mL	4
9	2.22	Traumatic lesion	80	M	----	1
10	2.64	Traumatic lesion	48	M	Ethanol: 0.29 g/L	3
11	7.23	Undetermined	79	M	Sertralina 118 ng/mL; Trazodone 168 ng/mL	1
12	7.29	Natural death	76	M	----	3
13	6.89	Traumatic lesion	22	M	----	0
14	20.51	Traumatic lesion	18	M	Ethanol: 0.35 g/L	0
15	22.58	Traumatic lesion	20	M	Ethanol: 0.28 g/L	1
16	7.28	Undetermined	0	M	Ethanol: 1.59 g/L	1
17	5.47	Natural death	62	F	Zolpidem < 100	1
18	3.07	Traumatic lesion	69	M	----	1
19	9.07	intoxication by CO	55	M	----	1
20	4.07	Undetermined	48	M	Ethanol: 0.33 g/L; THC-COOH 17.20	2
21	10.01	Traumatic lesion	57	M	Ethanol: 0.92 g/L	2
22	8.06	Natural death	74	F	Bromazepam: 17.90 ng/mL	1
23	8.47	Undetermined	45	F	Ethanol: 0.72 g/L; Ciamemazine 246 ng/mL	0
24	7.75	Traumatic lesion	23	M	----	3

25	15.80	Natural death	50	M	Tiapride 312 ng/mL; Diazepam 53.8 ng/mL; Nordiazepam 12.7 ng/mL; Temazepam 1.28 ng/mL	2
26	36.88	Natural death	54	M	ethanol: 0,18 g/L	1
27	9.72	Traumatic lesion	32	M	Clozapine 783 ng/mL; Lorazepam 1.33 ng/mL; Diazepam 392 ng/mL; Nordiazepam 392 ng/mL; Oxazepam 9.43 ng/mL; Temazepam 8.73 ng/mL	1
28	5.02	Traumatic lesion	14	M	----	1
29	7.80	Traumatic lesion	59	M	----	1
30	8.74	Natural death	46	M	Ethanol: 1,09 g/L; Trazodone 530 ng/mL; EDDP 71.3 ng/mL; Methadone 522 ng/mL; Flurazepam 101 ng/mL; Desalquilflurazepam 79.1 ng/mL	2
31	11.57	Natural death	0	M	Ethanol: 4,06 g/L; Desalquilflurazepam 24,3 ng/mL	1
32	6.49	Natural death	64	M	----	1
33	7.17	Natural death	46	F	----	1
34	3.72	Natural death	46	F	11-OH-THC 1.64 ng/mL; THC 3.46 ng/mL; THCCOOH 13.80 ng/mL	0
35	6.89	Traumatic lesion	67	M	----	1
36	9.99	Traumatic lesion	21	F	----	2
37	9.99	Natural death	76	F	----	2

WB – Whole blood

*PMI interval was calculated between death confirmation and autopsy.

Figure 1 - a) Chemical Structure of derivatized GHB b) Chemical structure of derivatized GHB-D_6

Figure 2 - MSMS Chromatogram of a sample spiked with 0.1 mg/L of GHB and 1.0 mg/L of GHB-D_6

Table S1

Ponderation factors behaviour.

Transformation factor	r^2	Σ of residues (%)	a	b	$S_{y/x}$
1 (not transformed)	0.9914	196.153	-0.0122	0.000070	0.023604
1/x	0.9916	109.575	-0.0041	0.000071	0.024869
$1/x^2$	0.9824	91.902	-0.0010	0.000067	0.037504
$1/x^{1/2}$	0.9927	146.666	-0.0080	0.000073	0.023821
1/y	0.9908	109.854	-0.0041	0.000071	0.025635
$1/y^2$	0.9794	97.440	-0.0008	0.000064	0.047794
$1/y^{1/2}$	0.9926	146.565	-0.0080	0.000073	0.023877