Review Article

Gamma-Hydroxybutyric Acid endogenous production and post-mortem behaviour – The importance of different biological matrices, cut-off reference values, sample collection and storage conditions

André L. Castro[a,b,*], M.Sc. (Forensic Toxicologist), **Mário Dias** [a,b], M.Sc. (Forensic Toxicologist), **Flávio Reis** [c,d], M.Sc., PhD (Pharmacology Researcher), **Helena M. Teixeira** [a,b,d,e], M.Sc.. PhD (Professor of Forensic Toxicology)

[a]National Institute of Legal Medicine and Forensic Sciences, Forensic Chemistry and Toxicology Service

[b]CENCIFOR – Forensic Sciences Centre, Coimbra, Portugal

[c]Laboratory of Pharmacology & Experimental Therapeutics, IBILI, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

[d]Faculty of Medicine, University of Coimbra, Coimbra, Portugal

[e]Faculty of Medicine, University of Porto, Porto, Portugal

*Corresponding author:

Address: National Institute of Legal Medicine and Forensic Sciences, Forensic Chemistry and Toxicology Service, North Branch, Jardim Carrilho Videira, s/n, 4050-067 Porto, Portugal

Phone: +351 222 073 850

Fax: +351 222 018 069

E-mail address: andre.castro@inml.mj.pt

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 for recreational and "dance club scene" use, muscle enhancement purposes and drug-facilitated sexual assaults. Its endogenous context can bring some difficulties when interpreting, in a forensic context, the analytical values achieved in biological samples. This manuscript reviewed several crucial aspects related to GHB forensic toxicology evaluation, such as its *post-mortem* behaviour in biological samples; endogenous production values, whether in *in vivo* and *in post-mortem* samples; sampling and storage conditions (including stability tests); and cut-off reference values evaluation for different biological samples, such as whole blood, plasma, serum, urine, saliva, bile, vitreous humour and hair. This revision highlights the need of specific sampling care, storage conditions, and cut-off reference values interpretation in different biological samples, essential for proper practical application in forensic toxicology.

Keywords: Gamma-Hydroxybutyric Acid (GHB); endogenous and *post-mortem* behaviour; *cut-off* reference values; sample collection; storage conditions, forensic toxicology

1. Introduction

Gamma-hydroxybutyric acid (gamma-hydroxybutyrate; GHB) (Fig. 1) is known to be an endogenous, naturally occurring, short-chained fatty acid compound found in mammalian tissues, with wide distribution and action in several brain areas (including hippocampus, basal ganglia, hypothalamus, and *substantia nigra*) [1-16]. Although it was first synthesised in 1960, researchers rapidly found out that it was an endogenous compound. With more than 30 years of clinical use, both in Europe and in the United States, its illicit use includes recreational use, muscle building effects, and drug-facilitated sexual abuse, alone or mixed with other substances in beverages, due to its odourless and colourless liquid state [12-33].

An increasing consumption of GHB, as a muscle growth promoter and/or mild sedative, led to the public authorities concerned about its safety and effectiveness for licit use without clinical supervision. Although it has been used as an anaesthetic, for narcolepsy and alcohol dependence treatment, weight loss, mood enhancement, ageing prevention, anxiety and depression reduction, balding treatment, and even as an aphrodisiac agent, it was banned from the licit market in the nineties. However, this was not enough to avoid the product achievement through internet, by the street names: *liquid X, liquid ecstasy, grievous bodily harm, scoop, cherry meth, soap, salty water, organic quaalude and growth hormone booster* [1,16,22-23]. Nevertheless, besides being illicitly administered, it can be used after buying its legal precursors such as gamma-butyrolactone (GBL) or 1,4-butanediol, as they both rapidly turn into GHB once they enter the body [15,30,32,34-36]. The growing number of overdose cases and/or sexual assaults with the suspicion of GHB use (intentional or unintentionally) leaded to an increasing demand for toxicological analysis, and GHB analytical detection

in biological samples for forensic purposes became part of routine analysis in many toxicological laboratories.

The detected GHB concentrations, both in *in vivo* and in *post-mortem* samples, require a careful interpretation, not only due to its endogenous appearance, but also due to a possible *post-mortem* production, related to autolysis and microbial action phenomena, and also to its rapid metabolism and excretion. Thus, defining a specific cut-off value in biological samples is crucial, in order to distinguish external exposure from endogenous values [3,14-16,22,25-29,31,32,34-37]. In fact, GHB can be frequently detected from physiological to pharmacological concentrations, and it can also be detected at toxic concentrations in *post-mortem* blood samples, even when there is no suspicion of GHB use [14,30,38].

This review aimed to describe some important aspects of GHB pharmacology and forensic toxicology, such as the *post-mortem* behaviour in biological samples, the endogenous production values, *in vivo* or in *post-mortem* samples, sampling and storage conditions, and cut-off reference values in different biological samples.

2. Data collection Method

The described references were downloaded through "B-on", the Academic Public Portuguese search engine for science and scientific publications, which searches the following databases: BioMed Central, BioOne, Bioline International, DOAJ, Future Science Group, INFORMS, Medline / Pubmed, Project Gutenberg, PubMed Central, Public Library of Science (PLoS), Scielo Global, Universidade de la Rioja - DialNet, RCAAP, ACM Digital Library, America Chemical Society (via CrossRef), American Institute of Physics, Annual Reviews, Elsevier, IEEE, IOP Publishing (Institute of Physics), Nature Publishing Group, Royal Society of Chemistry, Sage Publications

(political and Sociologic), SIAM, SpringerLink, Taylor & Francis, Wiley, Web of Science, Academic Search Complete, American Chemical Society, Business Source Complete, Cinahl, Health Business Elite, Web of Knowledge and Zentralblath. The articles considered relevant for the issue reviewed in this manuscript were obtained using the following searched terms: "GHB", "forensic toxicology", "endogenous values", "GHB stability", "GHB *antemortem*" and "GHB *postmortem* behaviour". The titles and abstracts of the obtained communications were reviewed to determine whether the information was relevant. The final step involved reading each one of the manuscripts for the evaluation of the contained information.

3. GHB pharmacokinetic and pharmacodynamic profile

Considered as a distinct neurotransmitter and neuromodulator, GHB represents a unique pharmacological entity, although it shares some cellular and behavioural effects with classical sedative/hypnotics [1,24,31,38]. Considered to be a powerful and fast acting central nervous depressant, GHB has an half-life from 20 to 60 minutes, is extensively metabolized, and less than 5% of an oral dose is eliminated unchanged in urine [16,26-28,31,36]. Therefore, only very few hours remain between the victim awakening, full consciousness recovering, and the latest possible time for taking blood or urine samples as evidence. Exogenous GHB usually becomes undetectable in, more or less, 12 hours in urine and 6 hours in blood or plasma [12,15,27,29,31,33,36-39]. Thus, late sampling can be a problem and a reasonable justification for a possible underestimation of the total number of positive cases [12,15].

Suggested to be, at the same time, a metabolite and precursor of gamma-aminobutyric acid (GABA), GHB is heterogeneously distributed in the central nervous system (CNS), with higher levels in the hippocampus, basal ganglia, hypothalamus, and

substantia nigra [1-16]. The primary source of GHB in the brain results from the metabolism of GABA, which is first deaminated to succinic semialdehyde (SSA), by GABA aminotransferase. The majority of the produced SSA is converted to succinate by succinic semialdehyde dehydrogenase (SSADH), and incorporated into the Krebs cycle (Fig. 2). However, a small portion, less than 2%, is converted by a specific neuronal cytosolic enzyme, SSA reductase, to GHB. Some authors suggest that there are alternative sources of GHB, which may play a very significant role in GHB production, especially in the periphery where GHB levels are very high, even though the levels of GABA are very low or even absent. 1,4-Butanediol, a naturally occurring aliphatic alcohol, has been shown to be a source of GHB. The GBL, a natural lactone precursor, is readily and irreversibly metabolized to GHB, by peripheral lactonases. Both 1,4-butanediol and GBL are present in the rat brain, at a 1:10 ratio, when comparing to GHB [1]. Being a weak binder to $GABA_B$ receptors, GHB has its own receptors (GHB_A and GHB_B); nevertheless, some of the actions due to exogenous GHB consumption may be linked to this weak $GABA_B$ agonist activity [1,15,19,22,28,31,34,39].

Although GHB functions are still being studied and confirmed, it is generally accepted that GHB causes a dose-response increase or decrease on dopamine levels in the brain, affecting the cholinergic and serotonergic systems [31]. Once GHB is within the cell, it is metabolized by GHB-desidrogenase into SSA, followed by succinate and GABA. Succinate is further degraded into CO_2 and H_2O. The most reported feature of these reactions is that they proceed rapidly, with an half-life from 30 to 50 min, for GHB, in the body, being this half-life dose-dependent, due to the saturability of the elimination pathway, which provides a nonlinear elimination kinetics , leading to longer half-lives, whenever an overdose is concerned [1,19,22,23,28,34,36,38,39].

It is important to be aware that there is a rare genetic disorder called GHB aciduria, which results from a failure on semialdehyde dehydrogenase. The subsequent accumulation of GHB arouses, due to an alternative metabolic pathway, with the reduction of succinic semialdehyde by a NADPH-dependent succinic semialdehyde reductase. Endogenous urinary GHB values in these patients are reported to reach more than 200 mg/L, and thus, this possibility should not be excluded [19,34,39].

GHB may induce sedative and/or anaesthetic effects, with the last effect connected to higher doses. It is also associated with an amnesiac effect. However, the GHB-produced depressant effects profile is clearly different from other depressants, such as benzodiazepines or barbiturates. In animal models, it has been shown that GHB produces EEG changes, reminiscent of epileptiform patterns, suggesting that GHB induces a cataleptic state, rather than a true sedation [1,12,13,22,25].

Ravers and recreational consumers look for some of the GHB intoxication effects, such as euphoria, reduced inhibitions, sedation, sleep-induction and muscle relaxation post-ecstasy consumption [12,13,16,39]. At sufficiently high doses, GHB induces CNS excitation, eventually followed by myoclonic jerks and clonic seizures, inducing a dissociative state comparable to the one induced by ketamine. At lower doses, GHB has shown the increasing of slow wave sleep, and the increasing and consolidating of REM (Rapid eye movement) episodes, contributing for a clinical use in narcolepsy [23]. However, negative symptoms may appear, such as confusion, dizziness, vomiting, nausea, bradycardia, respiratory depression, amnesia, strong sedation, and even death [16].

4. Endogenous production and possible influences

GHB endogenous production can become a problem when a positive result interpretation is needed. Although endogenous GHB concentrations in blood are initially in the lower nanogram range, several studies during the last years have suggested some reference values: above 5 mg/L in blood samples and 10 mg/L in urine samples, both *in vivo,* may be considered as exogenous, but there are even some proposals to diminish these values. Nevertheless, due to the rapid metabolism rate and excretion of GHB, it is still very difficult to detect these values 6-12 hours after consumption, even though it may be questioned if the detection interval for exogenous GHB administration can be prolonged with an appropriate pre-analytical sample treatment, lowering these cut-off values [3,5,6,19-22,25,27,28,34,37-41].

As previously mentioned, GHB aciduria is a rare genetic disorder, characterized by a deficit in succinic semialdehyde dehydrogenase, with subsequent accumulation of GHB. Higher values, such as 100 - 200 mg/L, can be even found. Thus, in these cases, this syndrome should be excluded, with a second blood sample taken to obtain basal levels of GHB from the evaluated individual [28].

Table 1 describes some data from the studies that used *ante-mortem* samples in order to define reference values, distinguishing GHB endogenous production from external exposure.

Some drug interactions are not already clarified. There have been some researches relating the concentration of some antiepileptic drugs, namely valproate, phenobarbital, barbital and chlorpromazine, and GHB increasing concentrations, by the interaction in their metabolic pathways. It has been shown that valproate could block the SSAD enzyme, which converts succinic semialdehyde into succinic acid, along with GHB dehydrogenase potent inhibition action. Besides, it has also been demonstrated that this compound enhances the activity of glutamate decarboxylase, which generates

GABA from glutamate. However, valproate also inhibits GABA transaminase, which will block the increase of GHB concentrations by this pathway. All the referred compounds are inhibitors of one enzyme that converts SSA to GHB, and could lead to lower GHB concentrations. Thus, no medication effects on urine or blood GHB levels have been accepted as certain [31].

Variability between volunteers and non-users has also been studied. Smokers, non-smokers, drinkers and non-drinkers have been studied by Moriya *et al*., and no statistical significance was found between these groups [24]. The possible influence of diet has also been studied, and the obtained results have suggested that food intake appears to have no influence on endogenous concentrations of GHB, at least in urine, [34].

The segmental analysis of hair is important to prove the existence of an external intake, comparing the GHB concentrations detected in the proximal hair segments, with the basal ones, both obtained from distal segments of the same hair sample. [12,29]. It is important to have a three week interval between the consumption and the hair collection, allowing GHB migration along the hair shaft [12]. Besides other studies, Goullé *et al* [29], described a statistical difference between basal levels and consumption levels in an healthy male with a controlled GHB consumption study, with an increase of GHB concentration in hair contemporary to the compound intake. Detection of single exposure is also important for Drug.Facilitated Sexual Assault context confirmation. Kintz *et al*. [12] described a real case, with a clear difference between the basal value and the value obtained in the consumption time-window. Although the number of developed studies in hair is not numerous, it seems clear that basal values and external consumption values are distinguishable in every positive case.

Bertol *et al.* [46] studied the possible influence of hair colour and dyed hair procedures in endogenous GHB hair values. Unchanged values were found between the groups, which presented identical mean concentrations and range of values. The same authors, in a parallel study, with a controlled consumption by 12 volunteers, applied a segmental analysis procedure, which has shown a statistically significant difference between basal levels and consumption concentrations. Applying the same method to three real cases, the authors reinforced the existence of distinct exogenous and endogenous GHB contents [46].

4.1 Storage conditions

Some studies have been performed in order to evaluate the influence of storage conditions on the detected GHB concentrations and in fact, LeBeau *et al*. [22] observed that storage temperature does affect the increasing of endogenous GHB levels in urine, being proportional to the increase of the storage temperature. The same authors also suggested, in a long-term storage study, that this increasing could be due to time, temperature, and/or changes in pH, as some of the GHB could be transformed in GBL at a normal urine pH [25,31].

GHB maximum values and stability in non-users *ante-mortem* samples (plasma and/or whole blood) have been studied, considering storage temperature, use of preservatives, sampling time, storage time or delay between death and autopsy. Table 2 shows the results of the most relevant studies found on this issue.

Fjeld *et al* [30] reanalysed 19 *ante-mortem* whole blood samples, stored at -20ºC, between 0.4 and 5.7 years. Eleven cases showed an increase in GHB concentration, while 8 showed a decrease, and no correlation was found between the changing on each concentration level and the storage time. In addition, no significant

statistic change was observed in these GHB concentration changes during storage time. Also eight negative samples were reanalysed, and all of them kept negative.

5. *Post-mortem* behaviour

GHB *post-mortem* synthesis is also an important occurrence that must be taken into account when interpreting the results, since this compound continues being produced after death, and we must have an extreme caution when interpreting potential GHB-related fatalities. In fact, GHB has been found in *post-mortem* biological fluids, some of the cases in lethal doses, although there was absolutely no suspicion of its prior consumption [5, 12-14, 23, 25, 28, 30, 38].

Even though such findings have shown blood concentrations up to 200 mg/L, this data can have been influenced by storage conditions and by the specificity of the used analytical technique. In fact, some studies have shown that the presence or the absence of preservatives, as well as different storage temperatures, can be the reason for GHB concentrations increasing during time [5,38]. It has also been shown that GHB *post-mortem* production is not limited to blood samples, but can also be observed in other biological fluids and tissues (such as urine and vitreous humour), although in smaller proportions [5, 13, 25, 26]. Some authors suggest that this production may be due to usual cellular autolysis processes, with enzymatic conversion of GABA, succinic acid and putrescine (poliamine present in eucaryota cells with an active role in cell differentiation and proliferation). GABA is an intermediate product in a first phase, and GHB is another intermediate product, in a second phase. Both can even be totally consumed, whenever this metabolism is linked to a microbial action. Moriya and Hashimoto suggested that bacteria may metabolize glucose to succinic acid via phosphoenolpyruvate and oxaloacetate, with further entry in the GHB production

pathway [38]. Other studies also suggested that GHB production may be specific to different microbial species, namely *pseudomonas spp.*, bringing more difficulties when interpreting GHB concentrations, since they might be present or absent in the studied samples [5,14,23,26,30,38].

Extra complexity to forensic results interpretation can be given by the fact that GHB can rapidly be eliminated from blood, particularly if there is a survival time between ingestion and death, and these values reach the ones detected in endogenous *ante-mortem* cases, as well as those resultant of a *post-mortem* production [23].

In addition to the conventional blood and urine samples, the use of alternative ones, like vitreous humour, characterized by an increased structural integrity and simplified matrix components, can be an advantage. Moreover, it is important to be aware that GHB intoxication may lead, among other symptoms, to urinary incontinence, and thus, the absence of an urine sample can be a common situation [23].

Concentration in *post-mortem* samples can reach significant values, even if the individual is a non-consumer, being this production minimized by the use of sodium fluoride as a preservative in whole blood samples (1-5%), and sample storage at -20ºC[5].

Cut-off values of 30 mg/L and 10 mg/L were proposed, for whole blood and urine samples, respectively, as long as there are no signs of advanced putrefaction. The urine smaller value may be justified by the absence of microbial species, due to a lack of survival capacity, and by the absence of GABA and the enzymes involved in the GHB metabolism process [5]. Some studies have even concluded that, with samples immediately frozen after collection, no change in the GHB concentrations is observed for, at least, 50 months. Nevertheless, it was shown that there is a positive correlation between *post-mortem* time increasing (gap between death and samples collecting) and

GHB concentration in the same samples. On the other hand, Elliott *et al.* [5,14] didn´t observe a proportional relationship between GHB concentration and putrefaction extent [5, 14, 23, 25,26, 28, 42-44]. Table 3 shows some data about the GHB behaviour in *post-mortem* samples.

6. Stability studies

Beránková *et al*. [26] studied the GHB stability on *post-mortem* urine and whole blood samples, in subjects with no previous GHB consumption history , using samples kept at 4ºC, with NaF, and performed at months 2 and 4. During the first two months, GHB concentrations suffered an increase (up to 30 mg/mL), followed by a decreasing in the following two months. In fact, it has been shown that *in vitro* GHB production, during storage, is more substantial in *post-mortem* samples than in *ante-mortem* ones. Additionally, lower temperatures, and the use of preservatives (such as NaF) can be crucial in long-term storage samples [26].

Moriya and Hashimoto [38] suggested that there might be a correlation between GHB concentrations in whole blood and the corresponding *post-mortem* intervals, whereas no correlation between GHB concentrations and storage periods was observed, if stored at -20ºC. During ten days, a 1.51 ± 1.15 μg/mL increasing was achieved in 14 *post-mortem* samples stored at 4ºC. In another study, with 8 *post-mortem* samples stored at 4ºC, there was an increase of 1.72 ± 0.76 μg/mL Fjeld *et al* [30] studied and reanalysed 18 *post-mortem* whole blood samples, stored between 0.5 and 7.2 years, and concluded that three of the cases showed an increasing, while 15 had a decreasing in GHB concentrations. Although there was a significant statistically reduction on GHB concentration after storage, no correlation between the concentration changing and storage time was found. Paul *et al.* [32] studied the variation in six blank urines, stored

at -20ºC after 6, 12 and 24 months, and no significant statistically variations were found, when comparing with the values achieved in the same samples, analysed before 24 hours after collection. Marinetti *et al* [23] suggest that vitreous humour can be an interesting alternative sample to verify a possible *post-mortem* production, since the results seem to be similar to the ones achieved in urine. Those two samples might help the pathologist interpretation, as it may allow the differentiation between exogenous consumption and *post-mortem* production. On the other hand, Elliott [14] also found out that, in two non GHB related *post-mortem* cases, the concentrations detected in both urine and vitreous humour were also comparable, both under the suggested cut-off for external exposure.

7. Practical recommendations and concluding remarks

Specimens should be rather collected aseptically, which is only possible in *in vivo* samples. In fact, *in vivo* blood is usually bacteria-free. Thus, sample collection in asepsis conditions could help the avoidance of *in vitro* production. On the other hand, *post-mortem* samples start some of the degradation processes right after death. Putrefaction is also characterized by resurgence of hydroxybutiric acids, including GHB, and thus, bacterial contamination is unavoidable and unpredictable [28]. It is also suggested, in a more radical approach, that a complete inactivation of brain enzymes (e.g., by irradiation) at the time of death might prevent further GHB *post-mortem* production [14].

During the years, a decreasing in the suggested reference concentration values has been observed, both for urine and whole blood samples, which may be explained by the development on sampling and storage protocols, which help to avoid *in vitro* GHB production. On the other hand, it can also be due to a constant development and

application of further new and reliable detection and quantification methods. However, it should never be forgotten that the collection of a second urine sample must be performed, considering the possibility of GHB aciduria, allowing the achievement of an individual basal quantification value [19,34,39]. Nowadays, the current suggested cut-off values are between 5-10 mg/L for *ante-mortem* urine samples and up to 10 mg/L for *post-mortem* samples.

Several studies indicate that GHB is stable under different storage conditions, in blood samples from living subjects. However, in *post-mortem* blood samples, a stronger influence of preservation and storage temperature on endogenous concentrations has been observed [30]. Meanwhile, the cause of death (in non-GHB related deaths) has not yet shown any relationship on GHB *post-mortem* concentrations, both in urine and blood [14].

Fjeld *et al* [30] suggest that GHB concentration is stable for several years at -20ºC, with fluoride preservation, both for *ante-mortem* and *post-mortem* whole blood samples. Nevertheless, concentration range is so variable that it is suggested that, for forensic interpretation, analytical results should only be given after analysing different matrices (such as urine, hair and others) [30].

Nonetheless, samples must be maintained in refrigerated or frozen conditions and analysed as soon as possible, preferably promptly after collection. A preservative must be added, such as sodium azide, NaF or EDTA, which has shown, in *post-mortem* samples, the best results in terms of GHB concentrations. Sodium citrate should be avoided since led to higher GHB concentrations in *post-mortem* blood samples [28, 31, 38]. Although further investigation on in-life endogenous blood GHB levels is suggested, interpretative cut-off settings and preventive measures for *in vitro* production of GHB seems always needed for endogenous values *versus* GHB intake values

differentiation [28,31], even though the current suggestions between 3-5 mg/L already seems reasonable.

These established cut-off values, for urine and whole blood samples, relies mainly on empiric data, and the suggestion and acceptance of reference cut-off values for endogenous detection must always be used with extreme care. As an example, in most of the cited references, in tables 1 and 3, the suggested reference values start from the higher value achieved for each processed batch of samples, for *in vivo* [3,15,16,19,25,28,31,32,33,34] and *post-mortem* samples [5,13,14], in a conservative and defensive way of analytical results interpretation.

To obtain complementary data, the use of alternative matrices should also be considered, in order to better help an improve the toxicological interpretation of the analytical findings [29,36]. However, once again, the sample collection time seems crucial, since the detection window stays unchanged (maximum of 12 hours), in saliva and sweat. It is also important to state that sweat shows high concentrations of GHB, even in non-users, which must be considered when this matrix is analysed [12]. On the other hand, Kintz *et al* [13] reported that bile does not fit the requirements that allow the discrimination between endogenous and external exposure GHB concentrations, due to the large concentration range achieved in their study, suggesting that femoral blood and vitreous humour should be used, instead of cardiac blood, more prone to *post-mortem* GHB synthesis. Curiously, Elliott [14] has shown that the two cases using vitreous humour had comparable results to the same cases using urine samples.

The use of hair as an alternative sample allows a higher detection window and can be a useful option to urine and serum/blood samples. However, due to the low concentrations usually detected in this sample, the analytical procedures continue being a challenge to the toxicologist [36; the use of tandem mass spectrometry techniques and

a continuous development in extraction procedures, may allow some good results (Tables 2 and 3).

Some specific issues related to hair were already discussed. Kintz *et al.* [12], Goullé *et al* [29] and Bertol *et al.* [46] consider that the hair colour is not important, in terms of GHB basal level values, and also concluded that, in terms of consistency, basal levels of GHB in non-consumers hair may function as evidence. On the other hand, Kintz *et al.* [12] concluded that the individual gender is not significant for basal values, as GHB concentration is almost identical in male and female subjects, in a non-GHB user (2.21±0.57 and 2.47±0.69 ng/mg for males and females, respectively). On the other hand, beard should not be used, as GHB concentrations in this matrix are highly influenced by external contamination, namely by sweat [29,36]. Concerning the hair results interpretation, this matrix should be analysed twice (firstly, at the time of exposure and three to six weeks later), in order to determine GHB concentrations before, during, and after exposure, avoiding any sweat contamination. Moreover, it should also be analysed in a segmental procedure, allowing a range determination for each individual, as well as the detection of significant deviations in that same range of values throughout time [12,29,36]. The use of hair segmental analysis for single exposure detection and confirmation (e.g., in a DFSA case) seems to be almost mandatory. Kintz *et al.* and Goullé *et al.* showed significant differences in basal values and "exposure time-window" values. This approach could overpass the need of a cut-off reference value, whenever there is a suspicion of a single or non-regular exposure to the drug. Paul *et al.* [36], on the other hand, proved that it is important to have a perfect sample collection time, specifically concerning the time between the consumption and the hair collection.

In conclusion, the analysis of segmental hair for GHB quantitation improves the detection window of this compound, whenever there is a single-dose exposure (DFSA or others). It may also be possible to confirm the time-window described by the victim or consumer, comparing the values obtained in the different hair segments, and the use of the respective cut-off reference values, which may be individually applied to the individual, since endogenous values may be also detected in the same sample [47]. On the other hand, as mentioned in table 1, few studies have already addressed endogenous or exogenous GHB values in hair, suggesting that more studies must be developed. Sampling delay is also mandatory to a representative result of consumption reality, as referred by Paul *et al*., concerning that the high GHB values in the hair root are not a sign of GHB consumption.

Recent studies suggest the possibility to use metabolites as consumption biomarkers. However, until now, no GHB glucoronated metabolites were known [48]. In fact, Petersen *et al*. [48] have recently published a study describing a new GHB metabolite: GHB-glucuronide (GHB-GLUC). They have tested the use of this metabolite as a biomarker, in urine, however the results are not so promising, due to a large inter-individual variation of GHB-GLUC concentrations. Nevertheless, there is still a big lack of information on this compound, and different approaches must be tested or verified (use of time-profile studies in hair, relative concentrations with creatinine in urine, etc...) in order to prove the potential biomarker function of this new metabolite. At the same time, the referred metabolite is also pointed as a possible cause for the GHB concentrations variations through time, although no testing is still published on this [48].

Concerning drug interactions, they are still under discussion. Nevertheless, the use of valproate medication should lead to a very careful data interpretation, along with

the inborn defect of 4-aminobutyrate metabolism at least until valid data for GHB levels in urine and serum/blood for humans under this compound influence are available [31].

There is also a case report concerning the concomitant therapeutic use of sodium oxibate and topiramate. In a patient using GHB in a twice-nightly prescription for some time, it was added topiramate. After the first dose of topiramate, it was noticed a 2.8 fold increase of GHB concentration than without topiramate, leading to a coma state, reversed and with a rapid onset, following the stop of topiramate administration. In light of published information, possible interactions should be evaluated using formal pharmacokinetic studies.[49]

Finally, it should always be taken into account that any suggested threshold must be considered as an aid to all data interpretation, as to any forensic case is concerned, and not be seen as a rigid requirement. Thus, considering the complexity of the issue, an individual evaluation is always needed [31].

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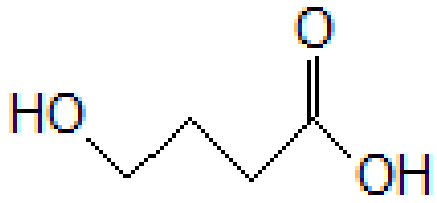

FIGURE 1: Chemical structure of Gamma-Hydroxybutyric acid.

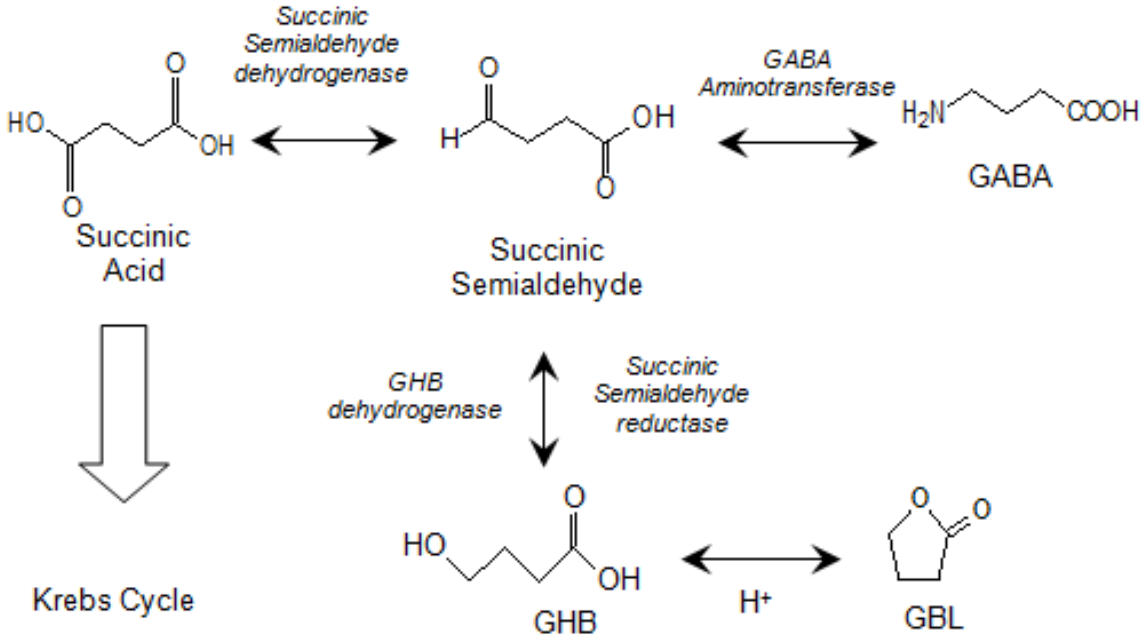

FIGURE 2: GHB formation and metabolism.

TABLE 1: Values of GHB obtained in several studies in antemortem samples

Ref	Casework	[Blood] in mg/L	[Urine] in mg/L	[hair] in ng/mg	[Saliva] in mg/L
	A – Blood, urine and saliva samples				
3	670 urine and 240 whole blood samples	5 (0.17 – 151)	10 (0.34 – 5.75)		
5	Author's own blood and urine	4	10		
15	50 non GHB-consumers women		5 (0.1 – 1.46)		
16	60 non GHB-consumers		3-10 (0.43 – 1.45)		
16	30 GHB consumers		3-10 (2.75 – 91.73)		
19	30 healthy volunteers		10 (0.16 – 2.14)		
19	20 diabetic volunteers		10 (0.17 – 3.03)		
34	119 volunteers non consumers		10 (n.d. – 3)		
34	15 volunteers non consumers	3 in plasma (< 2.5)			
24	20 volunteers		Male Smokers (0.52±0.37) Male non-smokers (0.28±0.21) Male drinkers (0.23±0.04) Female non-drinkers (0.29±0.12)		
28	24 healthy subjects in aseptically conditions	1 (0.005 – 0.010)			
31	50 urine and 50 serum samples from non-GHB users	4 (0.62 – 3.24)	6 (0.64 – 4.20)		
35	196 samples, from police controls in roadside testing	4	5-10		
32	6 blank blood and urine samples	5 (0.5-2.3)	10 (0.3 – 6.0)		
33	55 non-users urine samples		10 (0.9 – 3.5)		
37	Saliva (120 samples)				No cut-off (0.15-3.33)

Main remarks:

- Blood: Cut-off value of 4 mg/L, varying from 1 to 5 (reported individual values between 0.005 and 151).
- Urine: Cut-off value of 10 mg/L, varying from 3 to 10 (reported individual values between 0.1 and 91).
- Saliva: Only 1 study, without indication of cut-off value (individual values between 0.15 and 3.33)

	B – Hair samples	
12	Hair (real case – DFSA)	2.7
12	Hair (8 specimens male non-GHB users)	(0.5 – 12.0)
12	Hair (16 specimens female non-GHB users)	(0.5 – 12.0)
12	Hair (30 specimens non-GHB users)	(0.53 ± 0.20)
12	Blond Hair (8 samples of non-GHB users)	(0.5 – 12.0)
12	Brown Hair (6 samples of non-GHB users)	(0.5 – 12.0)
12	Black Hair (10 samples of non-GHB users)	(0.5 – 12.0)
29	Blond Hair (12 samples of non-GHB users)	1 (0.35-0.95)
29	Brown Hair (30 samples of non-GHB users)	No cut-off (0.41-1.86)
29	Black Hair (19 samples of non-GHB users)	No cut-off (0.32 – 1.54)
29	Hair (real case – illicit consumption)	14.0
29	Basal levels (12 individuals, non GHB-users)	No cut-off (0.31 – 8.4)
29	Hair (real case – DFSA)	No cut-off (basal: 0.71; Consumption: 3.1; 5.3; 4.3)
36	Hair (real case – recreational drug user)	No cut-off (basal 0.11-0.3; consumption: 3.81 ; 4.5)
45	Hair (real case – suspicion of consumption	After consumption stopping 1.20 – 1.49 During chronic consumption 4.74 -10.34
46	Basal levels (30 individuals, non GHB-users, n=10 for each hair colour)	Black hair: 0 – 4.49 Blonde hair: 0.58 – 5.09 Died hair: 0.61 – 4.02
46	Positive levels (12 individuals, single GHB-controlled consumption)	Basal level: 1.27 ± 0.73 One month: 5.35 ± 1.36 Two months: 4.62 ± 1.69

46	Three real cases	Case 1: 4.96 Case 2: 6.41 Case 3: 4.19

Main remarks:

- Hair: No clear indication of Cut-off values, varying from 1 to 14 ng/mg (reported individual values between 0.11 and 14).

Firstly, it is described the suggested cut-off values by each article author's, when referred. Secondly, in brackets, range of values obtained in each referred study.

TABLE 2: Values of GHB after stability, sampling and storage time and delay studies

Ref	Casework	[GHB] in mg/L	Storage temperature	Sampling delay	Storage time	Preservative addition
22	Two non-GHB consumers volunteers	Max: 0.52	-10ºC	24 H	180 days	X
22	Two non-GHB consumers volunteers	Max: 0.74	5ºC	24 H	180 days	X
22	Two non-GHB consumers volunteers	Max: 1.21	25ºC	24 H	180 days	X
25	After long-term storage	Max: 8.27	5ºC	< 7 days	189 days	X
27	15 ante-mortem plasma samples	Max: 2.5	4ºC	3 days		X
27	50 ante-mortem whole blood samples	Max: 1.56	4ºC	2 days		X
27	ante-mortem serum samples	Max: 3 µg/mL	4ºC / -20ºC	2 days		NaF
27	ante-mortem whole blood samples	Max: 13.1	---		36 months	Citrate Buffer
28	20 ante-mortem whole blood samples	0.005-0.010	-20ºC		Three hours	Aseptically collected No preservatives
28	3 Ante-mortem whole blood samples	Day 0: 0.008 Day 14: 0.017	-20ºC	14 days		0.1 % EDTA
28	3 Ante-mortem whole blood samples	Day 0: 0.008 Day 14: 0.016	4ºC	14 days		0.1 % EDTA + NaN_3 (0.1%)
28	3 Ante-mortem whole blood samples	Day 0: 0.008 Day 14: 0.020	4ºC	14 days		0.1 % EDTA
28	3 Ante-mortem whole blood samples	Day 0: 0.008 Day 14: 0.052	Room temperature	14 days		0.1 % EDTA
28	3 Ante-mortem whole blood samples	Max: 0.03	-20ºC		70 weeks	
28	3 Ante-mortem whole blood samples	Max: 0.4	4ºC		70 weeks	With and without NaN_3
39	100 ante-mortem urine samples	Max: 7	Room temperature		12 months	NaF (1%)

Main remarks:
- Storage: Mainly 4ºC (-20ºC also indicated).
- Sampling delay for no more than 14 days (less is better) and storage time at least for 180 days.
- Addition of preservative, being EDTA (0.1%) the most used.

TABLE 3: Values of GHB obtained in several studies in *post-mortem* samples

Ref.	Casework	[Blood] in mg/L	[Urine] in mg/L	[Bile] in mg/L	[Vitreous humour] in mg/L
5	*Post-mortem* (putrefied samples)	30 (2-29)	20 (0-18)		
23	96 *Post-mortem* (no history of GHB use)	(1.6-48)	(0-14)		
23	17 *Post-mortem* (no history of GHB use)	(3-107)	(1-7)		(1-6)
23	26 *Post-mortem* (after long-term storage)	(7-119)	(ND-7)		(ND-7)
28	15 *post-mortem*	(- 168)			
28	5 *Post-mortem* (no history of GHB use)	(0.4-2.6 μg/g)			
38	40 *post-mortem* non-users	(1.33-44.3)			
13	71 *post-mortem* cases (non-GHB related individuals)	Cardiac blood (0.4-409) (10-40 in majority)			
13	5 *post-mortem* cases (non-GHB related individuals)	50 (Femoral blood 16.8 – 44.1)			
13	6 *post-mortem* cases (non-GHB related individuals)				50 (3.9 – 21.4)
13	7 *post-mortem* cases (non-GHB related individuals)			6.1 – 238	
13	1 *post-mortem* GHB and heroin overdose real case	12		57	84
13	1 *post-mortem* GHB overdose real case	66 heart blood 77 femoral blood			85
14	38 unpreserved blood samples from *post-mortem* cases (non-GHB related death-causes)	30 (2-29)			
14	17 sodium fluoride preserved blood samples from *post-mortem* cases (non-GHB related death-causes)	30 (4-25)			
14	39 unpreserved urine samples from *post-mortem* cases (non-GHB related death-causes)		20 (0-18)		
14	15 sodium fluoride preserved urine samples from *post-mortem* cases (non-GHB related death-causes)		20 (0-10)		
14	2 unpreserved urine samples from *post-mortem* cases (non-GHB related death-causes)		(3-5)		(1-3)
1	GHB overdose real-case	648 heart blood 330 femoral blood			Hair: 47.4 in root bulb

Main remarks:

- Blood: Cut-off value of 30 mg/L, varying from 12 to 50 (reported individual values between 0.4 and 648).
- Urine: Cut-off value of 20 mg/L in all studies with indication (reported individual values between 0 and 18).
- Bile: Cut-off value of 57 mg/L in only one study (individual values between 61 and 238, one study only).
- Vitreous humour: Cut-off value of 84/85 mg/L, varying from 50 to 85 (individual values between 1 and 85).

Firstly, it is described the suggested cut-off values by each article author's, when referred. Secondly, in brackets, range of values obtained in each referred study.