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Review

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Hair testing of GHB: an everlasting issue in forensic toxicology

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Abstract

Background: In this paper, the authors present a critical review of different studies regarding hair testing of endogenous γ-hydroxybutyrate (GHB), concentrations in chronic users, and values measured after a single GHB exposure in drug facilitated sexual assault (DFSA) cases together with the role of a recently identified GHB metabolite, GHB-glucuronide.

Content: The following databases (up to March 2017) PubMed, Scopus and Web of Science were used, searching the following key words: γ-hydroxybutyrate, GHB, GHB glucuronide, hair. The main key words "GHB" and "γ-hydroxybutyrate" were searched singularly and then associated individually to each of the other keywords.

Summary: Of the 2304 sources found, only 20 were considered appropriate for the purpose of this paper. Summing up all the studies investigating endogenous GHB concentration in hair, a very broad concentration range from 0 to 12 ng/mg was found. In order to detect a single GHB dose in hair it is necessary to commonly wait 1 month for collecting hair and a segmental analysis of 3 or 5 mm fragments and the calculation of a ratio between the targeted segment and the others represent a reliable method to detect a single GHB intake considering that the ratios presently proposed vary from 3 and 10. The only two studies so far performed, investigating GHB-Glucuronide in hair, show that the latter does not seem to provide any diagnostic information regarding GHB exposure.

Outlook: A practical operative protocol is proposed to be applied in all suspected cases of GHB-facilitated sexual assault (GHB-FSA).

Keywords: drug facilitated sexual assault (DFSA), forensic toxicology; GHB-glucuronide; hair; γ-hydroxybutyrate (GHB).

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Introduction

The use of hair as an alternative and/or complementary matrix has increased in toxicological investigations with several clinical and forensic applications including: workplace drug testing, drug treatment programs, drug-facilitated sexual assault (DFSA), drug compliance testing and drug abstinence monitoring, long-term drug exposure in living and in post-mortem cases. Hair is a particular matrix as no active drug metabolism or excretion occurs within its structure once drugs have been deposited, unless cosmetic treatments can partially degrade the incorporated drugs [1, 2].

This is not the only issue to be evaluated when interpreting hair analysis, but also the risk of passive exposure/contamination for the external presence of drugs in hair or the presence of endogenous compounds, which can also be exogenously administrated. In this latter case the sole presence of the substance in hair is insufficient to confirm exogenous administration [3, 4].

An important example of this latter occurrence is that of γ-hydroxybutyrate (GHB). GHB has a dual nature, endogenous and exogenous, which makes the proof of its eventual exogenous intake a complicated task. Indeed, trace amounts of GHB are produced in various tissues, including the brain, where it functions as both a precursor and a metabolite of the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) [5]. On the other hand, GHB finds a broad application not only as a recreational drug of abuse in different contexts and in DFSA cases but also as a therapeutic agent under the form of sodium oxybate for the treatment of narcolepsy, the treatment of alcohol withdrawal syndrome and the maintenance of alcohol abstinence [6].

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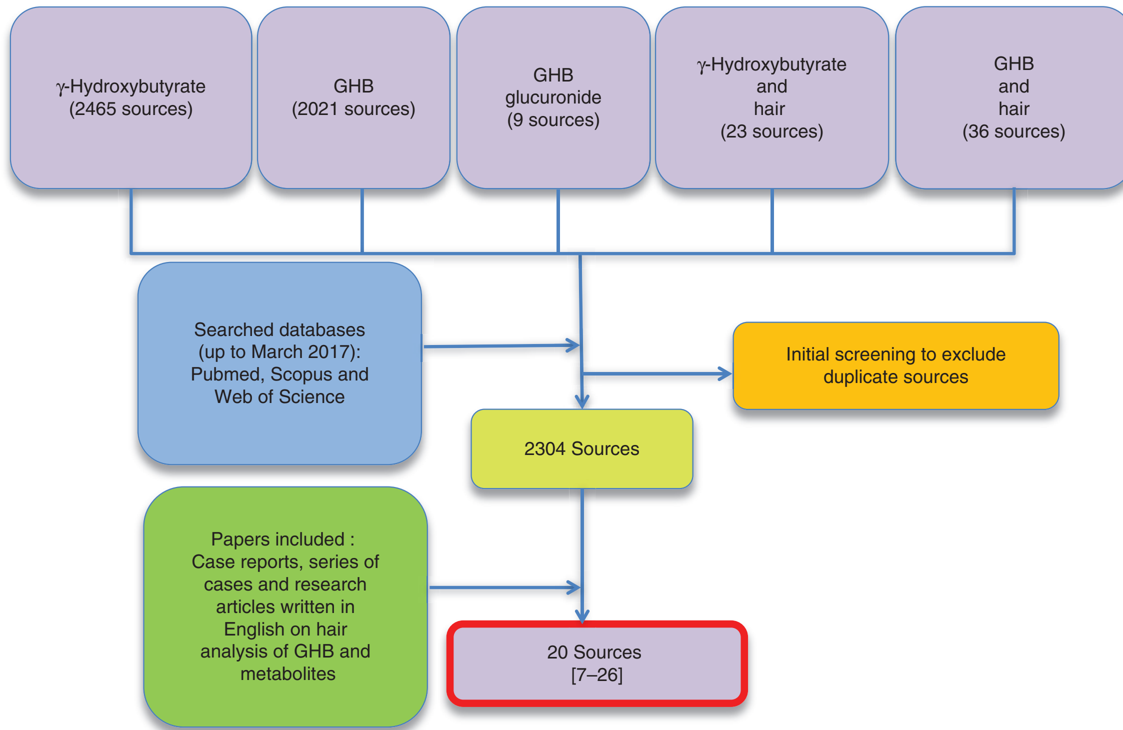

Figure 1: Flowchart of the selected papers and inclusion criteria.

The aim of this paper was to provide a critical review of the literature to compare the results obtained in different studies regarding GHB endogenous levels in hair, taking into account the role of hair color, possible cosmetic treatment, age, gender and eventual pathologies which can interfere with GHB values in hair. The authors also focused on hair analysis in GHB chronic users, on analytical approaches adopted for the detection of single GHB doses in hair especially in DFSA cases and on the role of recently identified new metabolites of GHB, in particular GHB-glucuronide.

Lastly, an overview of analytical methods for the determination of GHB in hair together with the extraction techniques used and the possible applications of these methods was provided.

After examining all these aspects, the authors drew exhaustive conclusions and provided possible future perspectives in the toxicological investigation of GHB in hair. A practical protocol to be applied in suspected cases of GHB-FSA has been also proposed.

Materials and methods

The following databases (up to March 2017) PubMed, Scopus and Web of Science were used, searching the following key words: γ-hydroxybutyrate, GHB, GHB-glucuronide, hair. The main key words "GHB" and "γ-hydroxybutyrate" were searched singularly and then associated individually to each of the other keywords.

Of the 2304 sources found, only 20 [7–26] were considered appropriate for the purpose of this paper. A flowchart of the selected papers and inclusion criteria are reported in Figure 1.

Results

GHB endogenous levels in hair

"As GHB is endogenous, GHB may be detected in hair at low concentration and as such, the presence of GHB alone is insufficient to confirm exogenous administration". This sentence has been reported in the Guidelines of Society of Hair Testing 2012 [27, 28]. However, no reference values of endogenous GHB in hair were provided in the same guidelines.

Literature searching has identified eight different studies [8, 9, 15, 16, 18, 20–22] where endogenous GHB values were investigated in hair. The first study by Kintz and collaborators in 2003, analyzed 24 hair samples

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collected from 8 males and 16 females [8]. They tested the first proximal 3 cm (from the root) of each of the 24 hair samples obtaining a range of values from 0.5 to 12.0 ng/mg. Moreover, they also evaluated if hair color and gender could affect endogenous hair GHB values, but no significant differences were found among hair strands of various colors (black [n = 10], mean: 2.37 ± 0.68 ng/mg; brown [n = 6], mean: 2.21 ± 0.71 ng/mg; blond [n = 8], mean: 2.44 ± 0.39 ng/mg) or in subjects of different gender (males, mean: 2.21 ± 0.57; females, mean: 2.47 ± 0.69 ng/mg).

Similarly, in 2003, Goullè et al. [9] measured baseline levels of GHB in 61 drug-free donors with results in the range of 0.32–1.86 ng/mg. Also in this case, it was found that hair color did not affect GHB content (blond hair [n = 12], 0.60 ± 0.19 ng/mg GHB, brown hair [n = 30], 0.90 ± 0.42 ng/mg GHB, black hair [n = 19], 0.90 ± 0.37 ng/mg GHB). In the same study, segmental hair analysis into 3 mm segments was performed in the other 12 volunteers (non-GHB consumers). In this case, a mean concentration of 1.22 ng/mg (range: 0.31–8.4 ng/mg) and a relative standard deviation for each individual ranging from 6.75% to 37.98%, was detected.

After these early investigations, recently further studies have been published in this field. Schröck et al. [15], obtained endogenous hair GHB in the range of 0.1–1.3 ng/mg examining hair samples of 27 GHB-free subjects with 8 of the 27 with concentrations below the limit of quantitation (LOQ) of 0.1 ng/mg hair.

In 2015, Bertol and Busardò and their respective research groups performed a study where hair samples of 30 volunteers were selected and divided according to color (10 samples of black hair, 10 samples of blond hair, and 10 samples of hair dyed with different colors) for the determination of endogenous GHB [16]. GHB was detected in the range between 0 and 5.09 ng/mg. In detail, black hair mean concentration was 2.11 ± 1.40 ng/mg with a range from <limit of detection (LOD) to 4.49 ng/mg; the blond hair mean concentration was 2.25 ± 1.31 ng/mg with a range from 0.58 to 5.09 ng/mg and the dyed hair mean concentration was 2.39 ± 0.97 ng/mg with a range from 0.61 to 4.02 ng/mg. Similarly to the studies of Kintz et al. [8] and Goullè et al. [9] there were no statistically significant differences in GHB concentration among the different hair colors. Moreover, no significant differences with respect to sex or age of the subjects were found.

In the same year, Bertol et al. [16] reported that, due to sweat contamination, GHB concentration in the first 0.5 cm hair segment of a group of non-consumers was statistically higher in comparison to all the other subsequent segments where mean GHB concentration was 1.27 ± 0.73 ng/mg.

More recently, Vaiano and colleagues [18] published another study where they investigated the role of age and gender on GHB baseline levels in hair and for this purpose a large number of subjects were included (n = 150). GHB was detected in the range of 0.27–2.84 ng/mg (mean: 0.71 ± 0.289 ng/mg), in accordance with previously published range values [8, 9, 15, 16]. Moreover, the authors highlighted that in males hair GHB concentration was significantly higher than in females: 0.829 vs. 0.596 ng/mg; $p < 0.05$.

This result was in contrast with their previous study, where it was not highlighted. Nevertheless, in this case a higher number of subjects was employed and the first cm of each hair sample was excluded to reduce the risk of sweat contamination as far as possible. Similarly to the results obtained by Vaiano et al., a significant difference ($p < 0.001$) between males and females was also detected by Shi et al. [20] when analyzing endogenous GHB in hair samples of 66 drug-free Chinese donors. The mean GHB value in males was 2.95 ng/mg (0.92–4.91 ng/mg, n = 35), whereas the mean value in females was 0.77 ng/mg (0.28–1.95 ng/mg, n = 31).

To demonstrate that endogenous GHB values in hair are not affected by post mortem enzymatic processes, Castro et al. [21] evaluated whole blood and hair post-mortem GHB values in 32 fatalities with previous information on death and autopsy data. Post-mortem interval (up to 5 days between death and sampling) affected GHB whole blood concentrations, but not that in hair samples where GHB was in a range of values similar to those of ante-mortem cases: 0.16–3.12 ng/mg (mean: 0.92 ng/mg).

Finally, in 2016 Kintz [22] documented a case of single exposure to GHB with a novel approach and he also collected hair samples from 12 non-consumers as a control group. The not decontaminated hair was cut into 6 × 1 cm segments. In the first cm, GHB values were in the range of 0.2–20 ng/mg, whereas in all the other segments it was always lower than 4 ng/mg.

All the above reported studies are briefly summarized in Table 1.

GHB long-term exposure in hair

There are few reports in the literature where chronic GHB exposure has been documented using hair. In 2005, a first French case of a fatal GHB overdose involving a 43-year-old man was studied by Kintz et al. [10] measuring GHB at very high concentrations in femoral and cardiac blood,

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Table 1: Brief overview of studies focusing on endogenous GHB concentrations in hair.

Hair color	Gender	N	GHB conc., ng/mg	Further information	Study/year/reference
Black, brown and blond	8 M + 16 F	24	0.5–12[a]	First 3 cm	Kintz et al. [8]
N.S.	M	8	2.21 ± 0.57[b]		
N.S.	F	16	2.47 ± 0.69[b]		
Black	M + F	10	2.37 ± 0.68[b]		
Brown	M + F	6	2.21 ± 0.71[b]		
Blond	M + F	8	2.44 ± 0.39[b]		
Black, brown and blond	M + F	61	0.32–1.86[a]	No hair segmentation	Goullè et al. [9]
Black	N.S.	19	0.90 ± 0.37[b]		
Brown	N.S.	30	0.90 ± 0.42[b]		
Blond	N.S.	12	0.60 ± 0.19[b]		
N.S.	N.S.	12	1.22[c] 0.31–8.4[a]	Hair segmentation (3 mm)	
N.S.	N.S.	27	0.1–1.3[a,e]		Schröck et al. [15]
N.S.	N.S.	30	0.0–5.09[a]	Hair segmentation (5 mm)	Bertol et al. [16]
Black	5 M + 5 F	10	0–4.49[a] 2.11 ± 1.40[b]		
Blond	5 M + 5 F	10	0.58–5.09[a] 2.25 ± 1.31[b]		
Dyed	F	10	0.61–4.02[a] 2.39 ± 0.97[b]		
Various colors	6 M + 6 F	12	1.27 ± 0.73[b]	Hair segmentation (5 mm) – The first 5 mm were excluded	
Various colors	75 M + 75 F	150	0.71 ± 0.29[b] 0.27–2.84[a]	The first cm was excluded	Vaiano et al. [18]
	M	75	0.83 ± 0.34[b]		
	F	75	0.60 ± 0.16[b]		
N.S.	35 M + 31 F	66	0.28–4.91[a] 1.93 ± 1.4[b]	The first 3 cm were analyzed	Shi et al. [20]
N.S.	M	35	0.92–4.91[a] 2.95[c]		
N.S.	F	31	0.28–1.95[a] 0.77[c]		
N.S.	23 M + 9 F	32	0.16–3.12[a] 0.92[d]	Post-mortem cases	Castro et al. [21]
N.S.	N.S.	12	First cm: 0.2–20[a] Other segments: <4	Hair samples were cut into 1 cm segments. Hair samples were not decontaminated	Kintz [22]

[a]Range. [b]Mean ± SD. [c]Mean. [d]Median. N.S., not specified. [e]In eight of the 27 hair samples the GHB concentrations were below the LOQ of 0.1 ng/mg hair.

urine, bile, vitreous humor, gastric content and pubic hair, as no head hair was available. Pubic hair was cut into three segments of 8 mm each and GHB was detected at the following concentrations: 25, 22.6 and 19.4 ng/mg, indicating a chronic GHB abuse.

In 2012 Bertol et al. [14] presented a case involving a 40-year-old man with a long history of GHB abuse and abstinence in the previous 2 months due to house arrest. A 17 cm long hair shaft was taken from the posterior vertex of the head and cut into 1 cm segments for the first 3 cm and then into 2 cm segments for the remaining proximal 14 cm. Ten different segments were obtained in total. The first three showed the following concentrations: 3.52, 1.49 and 1.2 ng/mg, whereas in the distal seven segments, GHB concentration ranged from 4.74 to 10.74 ng/mg. These GHB values allowed the authors to confirm that during the period in which the subject was under house arrest he did not use GHB whereas previously he was a chronic consumer and that the first hair segment showed higher GHB values for drug incorporation through sweat.

Mehling et al. [19] reported a DFSA case involving a 6-year-old female, who died following GHB sedation. GHB

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analysis was performed on a 16 cm hair shaft. The first proximal 4 cm of the hair were cut into 2 cm segments, whereas the next 12 cm were cut into segments of 1 cm length. The highest concentrations of GHB in the range of 13.6–40.8 ng/mg were found in the proximal seven hair segments and allowed to demonstrate a long exposure to GHB. When confronted with these results, the girl's uncle admitted the chronic administration of GHB to the child in the previous 8 months before death.

In the case of a 69-year-old man who was discovered dead at a friend's home, Jamey et al. reported a value of hair GHB in a 6 cm segment of 96.3 ng/mg suggesting a repeated consumption of this substance [23]. Toxicological analysis in peripheral blood also revealed the presence of 3-mmethylmethcathinone, pseudoephedrine together with GHB.

GHB single dose detected in hair

The identification of GHB exposure following a single dose administration has always represented a very difficult task for all forensic toxicologists dealing with this issue. As GHB has a very short detection window in conventional biological matrices, about 4–5 h in blood and 8–10 h in urine, if a longer time has elapsed after its ingestion there is little hope of detecting measurable amounts in these two matrices [5, 27]. Hence, some studies have focused on the detection of a single GHB dose in hair, which represents a crucial point especially in DFSA and other criminal cases [7–9, 15, 16, 20, 22].

One of the first studies [7] published in 2001 investigating GHB in hair, following a single dose administration, involved a 22-year-old female who had ingested a single GHB dose and died about 7 h later. Hair was plucked from the scalp. The root bulbs were removed, washed with sodium dodecyl sulfate and water and analyzed, whereas the hair shaft, also washed with sodium dodecyl sulfate and water, was cut in segments of 1.3 cm (0.5 inch). Segmental hair analysis showed no detectable amounts of GHB (LOD = 1 ng/mg), whereas hair root bulbs showed the following concentrations: 47.4 ng/mg for washed and 2221 ng/mg for unwashed hair, demonstrating that GHB is rapidly distributed into the hair follicle.

In a study published in 2003, Kintz et al. sought to investigate GHB hair concentration after a single intake [8]. For this reason, they administered a 25 mg/kg single dose of GHB to a 41-year-old man (weight 67 kg) and had available a DFSA case involving a 19-year-old female who had drunk a soft drink spiked with the drug. In both cases hair was collected 1 month after the single exposure. Hair samples were cut into 3-mm segments over a length of 3 cm (10 segments). Segmental hair analysis revealed in both cases GHB peak values, in the fifth segment for the administered subject and in the fourth segment in the DFSA case. The GHB peak values detected in the fifth and fourth segments demonstrated that a single exposure could be detected in hair (Figure 2A and B).

Goullè et al. [9] reported a case regarding a 29-year-old Black female raped under the influence of GHB, after drinking a glass of champagne. A 24-cm black hair shaft was collected and cut into 3-mm long segments (80 segments in total) 7 days after the rape and analyzed for GHB. The first three segments closer to the scalp showed the following GHB concentrations: 3.1, 5.3 and 4.3 ng/mg, whereas in the other 77 segments the mean concentration was 0.71 ng/mg (SD = 0.17 ng/mg). This mean value represented the baseline GHB level of the woman, whereas the highest GHB concentration detected in the second proximal segment was suggestive of an exogenous GHB administration even taking into account the possible contamination by sweat.

In contrast to the results of these two previous studies [8, 9] where a single GHB dose could have be identified in hair, Schröck et al. [15] found no increase in hair GHB after administering a single dose of 1.5 mL γ-butyrolactone (corresponding to 2.1 g of GHB, assuming a complete conversion). Head hair from to two volunteers was collected on days 9, 14, 25, and 28 for one case and on days 8, 17, 25, 37 for the other case. In both cases, head hair were segmented into 7 mm fragments. In addition, beard hair of 1 mm length was collected every day. GHB concentrations in head hair were in the range of 0.1–0.6 ng GHB per mg hair. In beard hair, the measured GHB concentrations were up to 20 times higher than those in head hair falling into the range of 0.2–3.1 ng/mg and 0.7–3.5 ng/mg, respectively, but no increased GHB values were observed for the first days after the intake of GBL. The explanation for these variations of GHB content in beard hair, provided by the authors, was a possible external contamination by sweat.

In all the above reported cases, what allowed the authors to confirm the administration of a single GHB dose in hair was the eventual rise of GHB content in targeted hair segments in comparison to the content in the others. However, none of these studies reported which had to be the degree of rise in hair concentration of GHB. Neither the Guidelines of Society of Hair Testing reported in 2012 the degree of this elevation [28]. They only stated that it had be "consistent with the approximate timeframe of the incident" in order to differentiate between endogenous and exogenous GHB.

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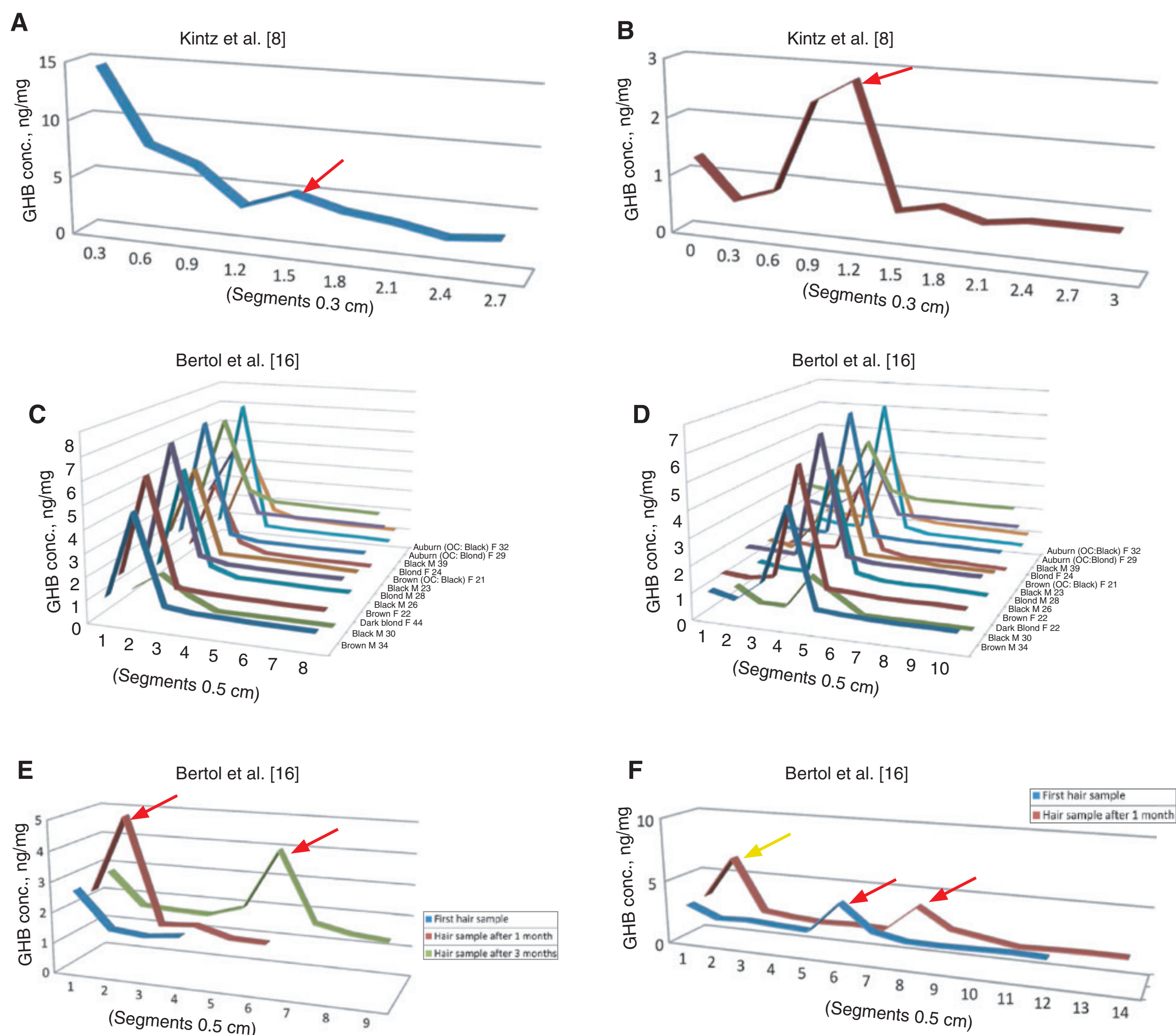

Figure 2: Cases of single GHB detection in hair from international literature.
In all cases (A–I) a segmental hair analysis to determine the "targeted segment" was performed.

In the Guidelines for the Forensic Analysis of Drugs Facilitating Sexual Assault and Other Criminal Acts [29], the United Nations Office on Drugs and Crime (UNODC) recommends that "the strand of hair has to be cut in 5–10 small segments (0.3–0.5 cm long) and each segment analyzed for GHB to identify if one segment has GHB concentration 10 times higher than the others, suggesting in this way possible administration of exogenous GHB". Four years after the release of this document, Bertol et al. [16] proposed lower ratios for GHB concentration in targeted segment vs. the others. The authors orally administered to 12 subjects a single 25 mg/kg GHB dose and hair shafts were taken 1 month and 2 months later and cut into 0.5 cm segments. Two ratios were obtained: 4.45:1 (95% CI 3.52–5.63) for GHB in the second segment in the hair samples collected after 1 month vs. the others and 3.35:1 (95% CI 2.14–5.18) for the fourth segment in the hair samples collected after 2 months vs. the others (Figure 2C and D), recommended for a positive identification of single GHB intake.

Indeed, Bertol et al. applied these two ratios to three DFSA cases, allowing the identification of a single exposure in two cases (cases 1 and 2) (Figure 2E and F) whereas for the third case it was not possible to draw exhaustive conclusions [16] (Figure 2G).

The two ratios proposed by Bertol et al. [16] were obtained from hair samples collected 1 and 2 months after a single GHB exposure, but after this time frame no information was available about eventual GHB concentration detectable in hair following a single dose when a longer period of time elapsed from drug intake. In this regard, Busardò et al. [25] monitored GHB peak concentration in a

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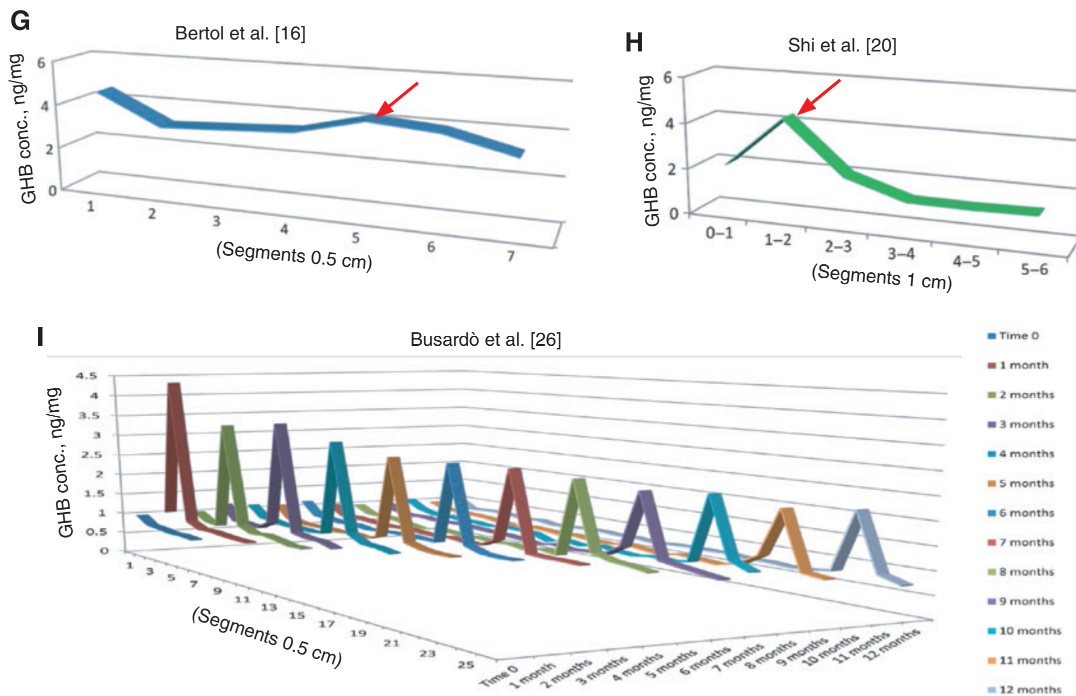

Figure 2 (continued)

targeted hair segment after a single DFSA administration over a period of 12 months. The same authors also calculated month by month the ratios between GHB value in targeted segments and that in the others for up to 12 months, showing a progressive decrease from 5.56 at the 1st month to 2.84 at the 12th month [25] (Figure 2I).

The month-by-month decrease of this ratio can be explained by the decay of hair GHB concentration, which occurred during the 12-month timeframe up to 51.6% at the 12th month. Moreover, differently from other drugs of abuse no significant GHB migration in the hair shaft was found.

Differently from the previous study, Shi et al. [20] reported another DFSA case involving a 20-year-old female where a 6 cm hair sample was collected 1 month after the assault and segmented into 1 cm fragments. High GHB concentration was found in the second segment (4.31 ng/mg) suggesting a GHB exogenous administration. However, no ratio with GHB values in the other segments was provided, making any definitive conclusion difficult (Figure 2H).

Different from all the above reported studies, is the approach to document a single GHB dose in hair, published by Kintz in 2016 [22], where two different cases were evaluated. The first was that of a subject administered a GHB dose of 25 mg/kg whose hair was collected 12 h after dosing and a 22-year-old female, a victim of a sexual assault, whose hair was collected 14 h after the assault. In both cases, hair samples were not decontaminated and cut into 6 × 1 cm segments. In the first cm the following concentrations were found: 1040 and 850 ng/mg, respectively, whereas in the remaining segments GHB concentration was always lower than 20 ng/mg. The comparison of the GHB values detected in the first hair segment of these two individuals with the ones of a control group of 12 subjects, in the range of 0.2–20 ng GHB per mg hair allowed Kintz to easily discriminate between endogenous and exogenous GHB. This new approach which is based on the analysis of proximal hair samples non decontaminated from eventual sweat contribution allowed Kintz to obtain a much better discriminating ratio which could be used in routine cases, considering that the risk of GHB passive contamination is limited because the drug is generally used in liquid formulation and not smoked [22].

GHB-glucuronide and other potential metabolites in hair

In 2003 Petersen et al. identified a new GHB metabolite in urine: GHB-glucuronide (GHB-glucuronide), with the hypothesis of using this metabolite as a biomarker for

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Table 2: Analytical methods applied for quali-quantitative analysis of GHB and metabolites in hair.

Sample weight, mg	Sample preparation	Analytical technique	LOD, ng/mg	LOQ, ng/mg	Linearity, ng/mg	Other analytes	Study/year/reference
10	Washing (1×5 mL 1% sodium dodecyl sulfate, 5×5 mL deionized water, 3 mL of methanol), water bath under alkaline conditions at 40°C for 2 h, SPE, derivatization	GC-MS	1	NA	NA	–	Kalasinsky et al. [7]
5–10	Washing (2×5 mL methylene chloride), cut, overnight incubation in 0.1 N NaOH, neutralization, LLE under acidic conditions, evaporation, derivatization	GC-MS/MS	0.1	0.2	0.2–20	–	Kintz et al. [8]
5	Washing (1×methanol, 2×hot water, 2×methylene chloride), cut, digestion under alkaline conditions, neutralization, LLE under acidic conditions, evaporation, derivatization	GC-MS/MS	NA	NA	NA	–	Goulle et al. [9]
25	Washing (acetone), digestion under alkaline conditions, neutralization, LLE, evaporation, reconstitution in mobile phase	LC-MS/MS	0.2	0.4	0.2–100	–	Stout et al. [12]
20	Cut, washing (methanol), overnight sonication in water, SPE, evaporation, derivatization	GC-MS/MS	NA	NA	NA	EtG	Paul et al. [13]
25	For GC–MS analysis: Washing (2×5 mL methylene chloride), cut, overnight incubation with NaOH, neutralization, LLE under acidic conditions, derivatization For LC-MS/MS analysis: Washing (2×5 mL methylene chloride), cut, rapid digestion with NaOH, LLE under acidic conditions, evaporation, reconstitution in mobile phase	GC-MS LC-MS/MS	GC–MS: 0.4 LC-MS/MS: 0.5	GC–MS: 0.6 LC-MS/MS: 0.6	GC–MS: 1–50 LC-MS/MS: 0.6–50	–	Bertol et al. [14]
20	Washing (water, acetone and methylene chloride), cut, ultrasonic bath in water, evaporation, reconstitution in 99:1 water: acetonitrile with 0.1% formic acid	LC-MS/MS	NA	0.1	0.1–10	–	Schröck et al. [15]
25	Washing (2×5 mL methylene chloride), digestion under alkaline conditions, LLE under acidic conditions, evaporation, reconstitution in mobile phase	LC-MS/MS	0.3	0.5	0.5–30.0	–	Bertol et al. [16]
25	Washing (1×methanol, 1×methylene chloride, 1×methanol), pulverization, digestion under alkaline conditions, neutralization, LLE, SPE, evaporation, reconstitution in mobile phase, filtration	LC-MS/MS	0.33	1.2	0.4–50	–	Jagerdeo et al. [17]
25	Washing (2×5 mL methylene chloride), cut, overnight incubation with NaOH, LLE under acidic conditions, derivatization	GC-MS	0.05	0.19	NA	–	Vaiano et al. [18]
20	Washing (deionized water and acetone), cut, digestion under alkaline conditions, neutralization, LLE under acidic conditions, derivatization	GC/MS/MS	0.02	0.05	0.05–15	–	Shi et al. [20]
10	Washing (1×1 mL isopropanol, 2×0.5 mL water), pulverization, incubation under acidic conditions, filtration, evaporation, reconstitution in deionized water, centrifugation	UHPLC/MS/MS	NA	0.32 for GHB (0.48 for GHB glucuronide)	LOQ 0.32–50	GHB glucuronide	Wang et al. [24]
25	Washing (2×2 mL methylene chloride), cut, incubation in M3 buffer, dilution with water	UHPLC/MS/MS	0.2 for GHB (0.2 for GHB glucuronide)	0.5 for GHB (0.5 for GHB glucuronide)	0.5–100	GHB glucuronide GABA GBL	Busardò et al. [26]

NA, not available; EtG, ethyl-glucuronide.

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GHB intoxication, by analogy with ethyl glucuronide as a biomarker of ethanol consumption. However, the results obtained in this first study did not look promising [30]. Only a few reports investigating GHB-glucuronide in different matrices [24, 26, 31, 32], were published after this study, two of which focused on hair [24, 26], Wang et al. [24] investigated the presence of GHB-glucuronide in the hair of 10 control subjects (laboratory staff) and in two GHB abusers. In the control group, GHB-glucuronide was found in the range of <LOQ (0.48 ng/mg) to 1.2 ng/mg, whereas in the two GHB users, GHB-glucuronide was only slightly elevated in 10 different 3 mm hair segments in one of the subjects (range 1.7–3.1 ng/mg) and below the LOQ in the other.

Busardò et al. [26] investigated one 10 cm head hair sample, from a 45-year-old man who had undergone sodium oxybate treatment for 6 months. After decontamination, the hair sample was dried and cut into 10 segments of 1 cm each and analyzed for GHB, GHB-glucuronide, GBL and GABA by UHPLC-MS/MS. The latter two compounds were not detected, whereas GHB was found in the first six proximal segments at significantly higher concentrations compared to the ones measured in last four segments, GHB-glucuronide was detected in all segments in the range: of 0.32–0.83 ng/mg, but no significant variation was found among the all.

In 2015 Hanisch et al. [33] investigated sulfonated metabolite of GHB (GHB-SUL) in urine samples on the reasonable assumption that it could be formed. The obtained results evidenced traces of GHB-SUL in urine, therefore indicating that GHB is not only glucuronidated but also sulfonated. To our knowledge no studies investigating GHB-SUL have been performed in hair so far.

Analytical methods for GHB analysis in hair

Several analytical methods have been published for GHB analysis in hair [7–9, 12–18, 20, 24, 26], using different techniques: GC-MS [7, 14, 18], GC-MS/MS [8, 9, 13, 20],

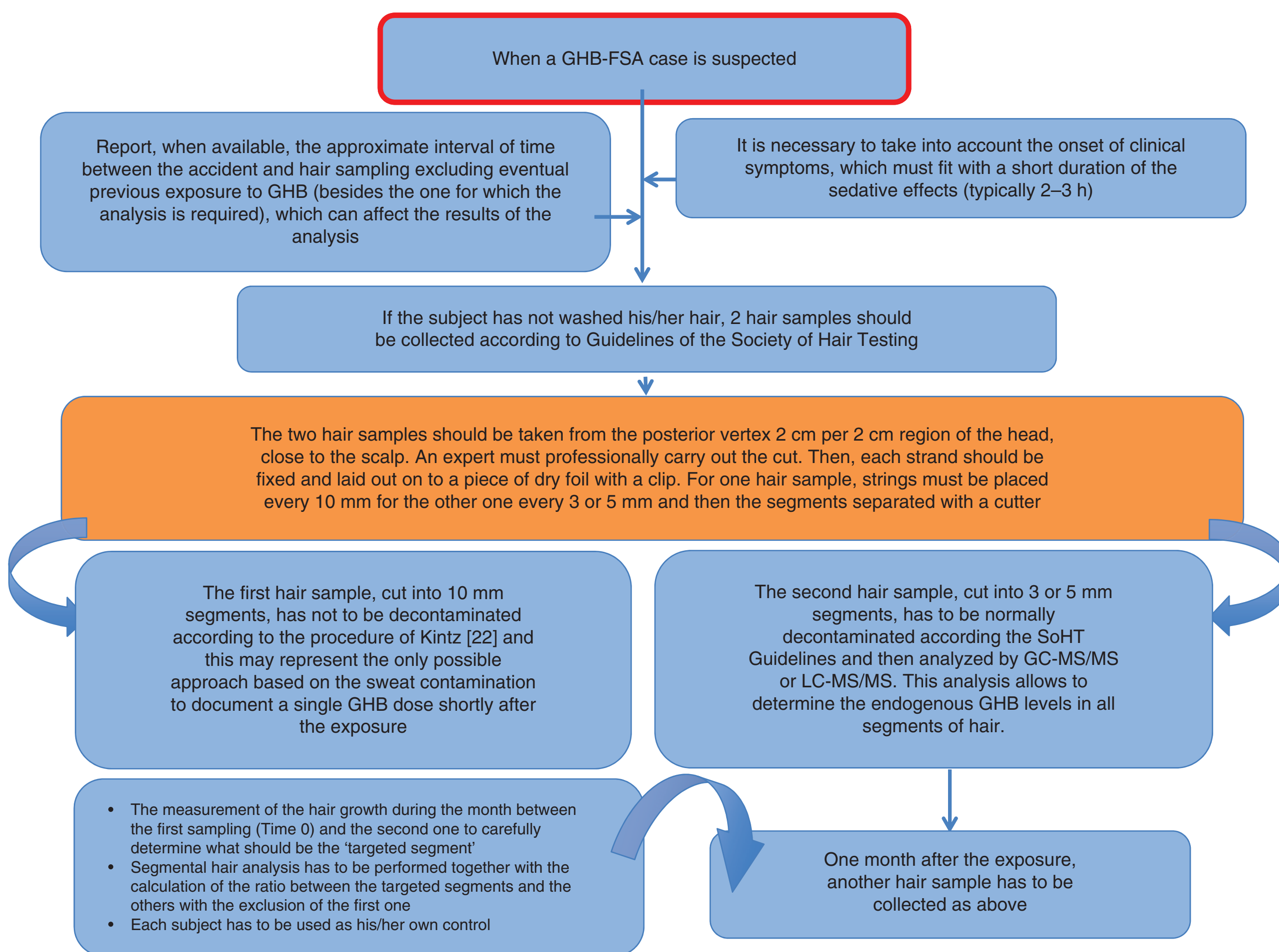

Figure 3: Practical protocol to be applied in all suspected cases of GHB-FSA.

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LC-MS/MS [12, 14–17] and UHPLC-MS/MS [24, 26]. The majority of these methods included only quali-quantitaive determination of GHB. Some others detected GHB with other compounds, such as ethyl-glucuronide [13], GABA, GBL [24] and GHB-glucuronide [24, 26].

Table 2 reports a brief overview of analytical methods applied for qualitative and quantitative analysis of GHB and metabolites in hair.

Conclusions and future perspectives

This comprehensive review allowed outlining some useful considerations:

- Summing up all the studies investigating endogenous GHB in hair [7–9, 15, 16, 20, 22] a very broad concentration range can be reported: 0–12 ng/mg. Moreover, considering the experiment carried out by Kintz [22] in non-decontaminated hair samples, the upper limit of this range reaches 20 ng/mg. Therefore, the high variability of GHB concentration in hair does not allow the proposal of a reliable GHB cut-off which can be applied unanimously to all cases to significantly discriminate between endogenous and exogenous GHB.
- The majority of studies have detected higher GHB values in the hair segment closest to the scalp, demonstrating the contribution from sweat even when hair was correctly decontaminated. In the light of this occurrence, at least the first 5 mm hair shaft should be excluded from analysis to avoid erroneous conclusions.
- All the studies investigating the detection of a single GHB dose in hair, except the one of Kintz [22] where a different approach is presented, indicate that it is necessary to wait a reasonable length of time for collecting hair (from a minimum of 7 days up to 1 month or more). The interval of 1 month from the possible exposure was mainly suggested to collect hair and this is the interval of time we recommend. The segmental analysis of hair into 3 or 5 mm segments and the calculation of a ratio between the targeted segment and the others represent a reliable method to detect single GHB dose in hair and the ratios proposed by Bertol et al. [16] for hair samples collected after 1 and 2 months can be applied in routine cases.
- Presently, the only approach which may allow to demonstrate a GHB ingestion shortly after the exposure is the one of Kintz [22], but it can be applied only if the subject has not washed his/her hair because it is based on the contamination property of sweat.
- The only two studies [24, 26] so far performed, investigating GHB-glucuronide in hair, highlighted that this metabolite does not seem to provide any diagnostic information regarding GHB exposure.

Finally, taking into consideration all the studies up to here discussed we wish to propose a practical protocol to be applied in all suspected cases of GHB-FSA, which is presented in Figure 3.

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