**Screening and confirmation methods for GHB determination in biological fluids**

**Ann-Sofie M.E. Ingels1,2, Sarah M. Wille2, Nele Samyn2, Willy E. Lambert1 and Christophe P. Stove1\***

*1Laboratory of Toxicology, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium*

*2Laboratory of Toxicology, National Institute of Criminalistics and Criminology, Federal Public Services, Vilvoordsesteenweg 100, B-1120 Brussels, Belgium*

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\*Corresponding author:

Christophe P. Stove

Ghent University

Faculty of Pharmaceutical Sciences

Laboratory of Toxicology

Harelbekestraat 72

9000 Ghent

Belgium

E-mail: Christophe.Stove@UGent.be

tel.: +32 9 264 81 21

fax: +32 9 264 81 83

**ABSTRACT**

The aim of this review is to provide a comprehensive overview of screening and confirmation methods reported to determine the low-molecular weight compound and drug of abuse gamma-hydroxybutyric acid (GHB) in biological fluids. The polar nature, the endogenous presence, rapid metabolization following ingestion and stability issues during storage (*de novo* formation and interconversion between GHB and its lactone form gamma-butyrolactone), impose challenges for the analyst and for the interpretation of a positive result. First, possible screening procedures for GHB are discussed, including colorimetric, enzymatic and chromatography-based procedures. Confirmation methods for clinical and forensic cases mostly involve gas chromatography (coupled to mass spectrometry), although also liquid chromatography and capillary zone electrophoresis have been applied. Prior to injection, sample preparation techniques include (a combination of) liquid-liquid, solid-phase or headspace extraction, as well as chemical modification of the polar compound. Also simple dilute-and-shoot may be sufficient in the case of urine or serum samples. Advantages, limitations and trends are discussed.

**KEYWORDS**

gamma-hydroxybutyric acid (GHB), gas chromatography - mass spectrometry (GC-MS), liquid chromatography – tandem mass spectrometry (LC-MS/MS), sample preparation

1. Introduction

Gamma-hydroxybutyric acid (GHB) or 4-hydroxybutanoic acid, a short chain fatty acid (pKa 4.6-4.8) with two polar substituent groups (hydroxyl- and carboxylgroup), was synthesized in the early sixties as a structural analogue of the neurotransmitter gamma-aminobutyric acid (GABA) that could cross the blood-brain barrier [1,2]. GHB was also found to be endogenously present in humans as a minor precursor and metabolite of GABA. Its role as endogenous compound remains unclear; it probably acts as neuromodulator or neurotransmitter through GABAb receptors and GHB specific receptors in the brain [3]. GHB can also be formed in humans from the precursors gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) (Fig. 1) [4,5].

The sodium salt of GHB, sodium oxybate, has been used therapeutically as an anaesthetic, but this use has been abandoned in most countries because of side effects such as seizures and amnesia [6]. Furthermore, GHB/GBL containing supplements were sold to increase body muscles - since it is assumed that GHB stimulates growth-hormone release - and to improve sleep, but these supplements have been withdrawn from the market since the late nineties after reports of abuse and side effects. Nonetheless, sodium oxybate is currently being used in the treatment of narcolepsy with cataplexy and excessive daytime sleepiness (Xyrem®) and of alcohol (Alcover®, Italy) as well as opiate withdrawal [7].

In addition, illegal GHB and its precursors, GBL and 1,4-BD, have become popular amongst clubbers because of their stimulating effects. The club drug is known under street names such as liquid ecstasy, Georgia Home Boy, Grievous Bodily Harm, soap, scoop and salty water [8]. GHB and its precursors are most commonly available as solutions, in small vials or mixed in bottles with e.g. water, from which typically a capful is ingested orally per dose [4]. GHB abuse has also been reported in drug facilitated sexual assaults (DFSA), because of its strong sedative and amnesic effects. The possibility to render a victim unconscious is enabled by the chemical properties of GHB, being a colourless liquid which can be easily mixed with other liquids [9,10].

A dose typically ingested for abuse ranges from 2 to 6 g GHB, corresponding to 25 to 75 mg/kg body weight. [5]. Euphoria, relaxation, increased sociability, and decreased psychomotor skills are amongst the effects experienced when using GHB, showing similarity with the effects reported for moderate alcohol intoxication [5]. GHB has a steep dose-response curve, with a narrow margin between therapeutic or desired and toxic effects [5,11,12]. More in particular, effects reported following recreational use of GHB and its precursors are dual, i.e. both sedative and stimulatory, depending on the dose*.* An individual dose of 1.0 to 2.0 g that is ingested orally results in effects like relaxation and euphoria, while doses of 2.5 to 3.0 g may lead to side-effects such as nausea and vomiting. Higher doses (3.0 to 4.0 g) may result in loss of consciousness, a dose of more than 4 g possibly resulting in respiratory depression and coma [13]. Finally, side effects of a severe intoxication may evolve to convulsions, coma, and death. Fatal incidents have been reported due to the use of GHB alone or in combination with other drugs such as alcohol and ecstasy [11].

First reports of abuse appeared in the early 1990s [12,14]. Since the late 1990s, early 2000s, the incidence of GHB intoxications has apparently been decreasing worldwide. For example, when evaluating GHB exposures reported to the California Poison Control System from 1999 to 2003, a decrease has been recorded, which can reflect the true incidence, but may also be due to a decrease in adverse events without a decrease in overall GHB usage or to random variability [14]. According to the annual reports of the latest drug situation and trends in the European Union and Norway, published by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), national estimates of the prevalence of GHB -where they exist- remains low. For example, in the UK, 2 % of regular *clubbers* reported last year use of GHB in an Internet survey [15]. Furthermore, GHB screening using automated clinical analyzers has not been routinely available until 2009. In addition, due to the rapid elimination of GHB from the human body, delayed sampling may result in GHB concentrations below the current cut-off values, thus no longer allowing proof of ingestion [16]. Therefore, true case incidence may be underestimated [5]. Moreover, more recent reports show that recreational use with limited awareness of the possibility to suddenly fall into a coma has resulted again in an increase in GHB intoxications at raves or other dance parties [11,17].

So, although general use may be relatively low compared to other drugs of abuse such as cannabis, amphetamines and cocaine, a higher use has been seen in subpopulations (e.g. men having sex with men), settings and geographical areas [18,19]. One should consider the possibility of a GHB-intoxication in the case of unexplained coma (in the absence of a head injury or elevated head pressure). Treatment of GHB overdose is primarily supportive, focussing on preservation of respiratory status, with no known antidote available [4]. Complete recovery has mostly been observed after 6 - 8 h, with a typical abrupt awakening of the patient [20]. Furthermore, following frequent ingestion (every 1 to 3 h, around-the-clock) dependence has been observed, as well as withdrawal symptoms such as agitation, anxiety, tachycardia, hypertension and delirium. These symptoms begin 1 to 6 h after the last dose and could last for 5 to 15 days [21,22].

Since the late 1990s, the Food and Drug Administration (FDA) banned all non-prescription sales of GHB and illicit GHB is since 2000 a Schedule I agent (Controlled Substances Act) in the US. The FDA has approved in 2002 the use of sodium oxybate for the treatment of narcolepsy with cataplexy (Xyrem®), so it has become available on prescription as a Schedule III agent. Also, in 2005, the European Medicines Agency (EMA) approved sodium oxybate for the treatment of narcolepsy with cataplexy. On the other hand, GBL is a List I controlled chemical, used for the manufacture of a controlled substance -GHB- , making its possession, manufacturing, or selling with the intention for ingestion illegal. The latter also applies to gamma-valerolactone (GVL) and 1,4-BD. So, the congeners of GHB are easily and moreover legally available on e.g. the internet, as long as the use is *not* intended for ingestion [4,14].

Various bioanalytical methods for GHB determination have been reported since the early 1970s [23-26]. This review will focus primarily on those methods published since the 1990s, when there was an emerging need for analytical methods to measure GHB in biological fluids as part of toxicological investigations, given the first reports of GHB abuse appearing in the US [27]. Also trends, advantages and disadvantages of sample preparation and analytical techniques are discussed. First, according to the generally applied strategy in toxicology, the so-called systematic toxicological analysis (STA), screening techniques including e.g. colorimetric and enzymatic tests will be discussed. These differentiate between (presumably) positive and negative GHB samples. Positive GHB results are then confirmed using a second, independent method, mostly involving quantitation [28]. This has been achieved mostly by gas chromatography (GC), although also liquid chromatography (LC) and capillary zone electrophoresis (CZE) have been applied.

2. Analytes of interest

Although in a toxicological context it might be relevant to determine whether GHB positivity is the result of the intake of GHB, GBL or 1,4-BD, GHB remains the most important analyte to search for in biological fluids, owing to the fast *in vivo* biotransformation of its precursors [29,30]. Also in fatalities involving consumption of large amounts of these precursors, high GHB and only low GBL and 1,4-BD levels have been observed [31]. Other compounds that might be of interest to determine simultaneously (in the same run) are the positional isomers and isobaric compounds alpha- and beta-hydroxybutyric acid (AHB, BHB; Fig. 1) (diabetic and post-mortem cases) [32-35], glycols (in emergency cases with coma of unknown origin when ingestion of GHB or ethyleneglycol (EG) is suspected, the latter also causing high anion gap metabolic acidosis; Fig. 1) [35-37] and other club drugs such as MDMA or ketamine [38], as well as gamma-hydroxyvaleric acid (GHV) or its lactone, GVL (reported to be a GHB alternative) [31,39] (Fig. 1).

It needs to be kept in mind that a quantitative result may be influenced by the *in vitro* interconversion of GHB and GBL in aqueous matrices, the equilibrium depending on pH and temperature [40]. Therefore, several methods have evaluated the rate of GHB/GBL conversion during sample treatment or analysis, with different outcomes. Overall, three scenarios have been described. First, conversion to either GBL or GHB was complete and was used for GHB determination [e.g. 8,41,42]. This implies that GHB is measured as “total GBL” (GHB converted to GBL + actual GBL present in the sample) or as “total GHB” (actual GHB present in the sample + GBL converted to GHB). In these scenarios, GHB, respectively GBL, is converted completely to GBL, respectively GHB, before analysis [8,41,42]. Secondly, conversion did not occur, so absolute GHB was measured [e.g. 16,35,36]; lastly, conversion occurred but was minimal, with little or no relevance in the forensic or clinical setting [e.g. 43,44]. Therefore, the method of analysis needs to be considered when comparing existing data from e.g. post-mortem analyses. In methods involving conversion of GHB to GBL (first scenario), slightly higher GHB concentrations may be observed (measured as “total GBL”, i.e. GHB converted to GBL + actual GBL present in the sample) than in methods determining absolute GHB. This may be due to the conversion of a proportion of the (endogenous) GHB present in a post-mortem plasma or urine sample to GBL during storage, depending on sample pH [45,46].

3. GHB concentrations and matrices of interest

As mentioned above, the natural presence of GHB results in measurable baseline levels in various biological matrices. Studies have been conducted in e.g. urine [e.g. 47-50], plasma [e.g. 49], serum [e.g. 50] whole blood [e.g. 32,48,49] and oral fluid [e.g. 51] samples obtained from healthy non-users. Also data from non-GHB related fatalities [e.g. 45,52], together with concentrations arising from exogenous administration have been collected. Ingestion can be intentional - for recreational or therapeutic use - or accidental, which both may lead to overdoses or even fatalities, illustrated by several case reports [e.g. 11,53-56]. Physiological concentrations of GHB, situated in the low and sub-microgram-per-milliliter range, are mostly well below concentrations found in intoxicated patients, where a narrow range exists between recreational doses and overdoses. An overlap between highly toxic and lethal concentrations has been observed, demonstrating high inter-individual variability between measured GHB concentration and effect [13,57]. According to the list of therapeutic and toxic concentrations from The International Association of Forensic Toxicologists (TIAFT), a value above 280 µg/ml of GHB in plasma may be sufficient to cause death [58]. In addition, elevation of GHB concentrations during storage of urine and blood samples has been reported, further complicating the interpretation of a GHB concentration. Therefore, an appropriate storage of samples until analysis is required (recommendation: - 20 °C), [9,13,46,57,59]. *De novo formation* has also been reported in post-mortem blood samples. For more detailed information concerning GHB production in post-mortem cases, we refer the interested reader to existing literature [45,52,60,61].

To differentiate between endo- and exogenous concentrations [49], cut-off levels have been established. Most authors agree on a 10 µg/ml cut-off level for GHB in ante-mortem urine [48,49,59], although suggestions of 5 [62] or 6 [50] µg/ml have been made as well. For ante-mortem whole blood, 10 [16,49], 5 [48] or 4 [50] µg/ml has been proposed as a cut-off, while one group even proposes 1 µg/ml, if appropriate storage is guaranteed [32]. This implies that screening and confirmation methods for GHB in ante-mortem urine, whole blood and plasma preferably have a decision limit or lower limit of quantification (LLOQ) below or equal to 4 or 5 µg/ml. Higher cut-off levels have been proposed for post-mortem matrices (20 for urine, 30 and 50 for whole blood and 12 µg/ml for vitreous humour) to exclude false positives [16,37,52]. For following up GHB concentrations in Xyrem® patients, a wide concentration range may be necessary, depending on the timing of sampling (shortly after intake *vs.* several hours later) [63]. Endogenous presence of GHB in various biological matrices not only renders true blank matrices unavailable for conducting method validation experiments, it also precludes the use of low calibrators (< 1 µg/ml) prepared in authentic matrices [33], and complicates the interpretation of a positive result.

As an alternative to the use of interpretative cut-off concentrations, continuous-flow GC-combustion-isotopic ratio MS has been used to discriminate between exogenous (i.e. synthetic) and endogenous GHB in blood samples. First findings suggest differences in the 13C and 12C content of the endogenous and synthetic form of GHB [64]. However, it is obvious that the cost and complexity associated with this high-end technique strongly limits its general applicability.

In addition to the endogenous presence and possible instability during storage, samples must be collected as soon as possible after ingestion, due to extensive metabolism of GHB once ingested orally (plasma T1/2 less than 1 h) [5,65,66]. Otherwise, the GHB level will drop in blood and urine to endogenous concentrations within 6 to 12 h following intake, no longer allowing to prove intake of GHB, possibly leading to an underestimation of the total number of positive cases [16]. Therefore, alternative sampling strategies and alternative matrices have been evaluated. These include dried blood spots (DBS), i.e. capillary whole blood obtained by fingerprick, facilitating sample collection, as well as non-conventional matrices such as sweat and oral fluid. Only moderate results have been obtained in the latter two matrices since diffusion of the acidic drug in these has been shown to be limited. Following GHB intake (50 mg/kg sodium GHB, n=5), only 1/4 to 1/3 of the concentration found in plasma was measured in oral fluid, with an even quicker return to baseline values and high oral fluid/plasma inter-variability, while in sweat, GHB concentrations were only slightly higher than baseline values [44,66-70]. On the other hand, hair analysis has been shown useful to extend the window of detection, because of incorporation of GHB in the hair matrix. A case report has described detection even after a single use in a case of DFSA [71]. Also in hair, endogenous GHB is present, often rendering it difficult to draw straightforward conclusions. Therefore, small segments are analyzed to detect an elevation of the baseline GHB concentration, due to exogenous ingestion [72,73].

More than 95 % of an oral dose of GHB is converted to CO2 and H2O as it enters the Krebs cycle *via* succinate, with less than 5 % being excreted ‘unchanged’ in urine [5]. Until recently, no specific metabolites of GHB were known. However, Petersen *et al.* (2013) [74] demonstrated the existence of a new metabolite, GHB-glucuronide, in urine, in concentrations ranging from 0.11 to 5.0 µg/ml. Although more research such as pharmacokinetic studies following GHB administration are required, this compound is theoretically a biomarker of GHB exposure with the potential to extend the window of detection in the conventional matrix urine [74].

4. Screening procedures for the presence of GHB in biological fluids

A good screening procedure allows the identification of unknown analytes in a simple, sensitive, selective and rapid way, starting from a minimal amount of sample. STA approaches typically utilize immuno- and/or enzymatic assays to screen for analytes or categories of compounds, next to GC-mass spectrometry (GC-MS) or high performance liquid chromatography- diode array detection (HPLC-DAD) for high-throughput screening for simultaneous detection of as many toxic compounds as possible. Liquid chromatography-mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) and high resolution techniques have been used to a lesser extent for such comprehensive screening but are gaining more and more interest nowadays, sometimes even replacing the immunological and/or enzymatic tests [28,75,76]. Below, an overview of possible screening procedures for GHB is given, starting with colorimetric tests. Given the lack of commercially available immunoassays, STA using chemical analyzers did not include GHB until 2009 [16,34]. Since then, an enzymatic assay adaptable to common analyzers has become commercially available (Bühlmann laboratories, Switzerland) [77]. Furthermore, several GC methods became available and recently LC-MS/MS-based methods have been reported with the focus on high-throughput, so both techniques can therefore also be used as screening tool. A screening method preferably has a decision limit (cut-off of the applied assay) at or below the exogenous/endogenous cut-off, to allow for a reliable first differentiation between samples considered to be GHB-positive or -negative. However, since moderately to severely intoxicated GHB patients such as those brought to an emergency department in comatose state will mostly display GHB concentrations well above these cut-off levels we also consider in this review methods with decision limits/LLOQs (well) above these cut-offs as screening methods. As with any screening test, a positive result should only be considered preliminary and needs to be confirmed using an independent, preferentially MS-based, technique such as GC- or LC-MS (/MS).

*4.1 Colorimetric tests*

Badcock and Zotti [78] reported a colorimetric test that allows the identification of GHB in human urine based on the conversion of GHB to GBL. Briefly, following the addition of concentrated sulphuric acid, ammonium sulphate and nitroprusside to 250 µl of urine, an intense and instant blue/olive-green colour will appear if GHB is present in the sample [78]. Another colorimetric test, a modification of the ferric hydroxamate test for ester detection, only requires 5 min to detect GHB in 0.3 to 1 ml urine, the presence of GHB being indicated by purple colouring of the sample [79]. Although both colorimetric tests are simple and results can be obtained in less than 10 min, the prime disadvantage is the lack of sensitivity, with limits of detection of 100 or even 500 µg/ml [78,79].

*4.2 Enzymatic assays*

Enzymatic assays to determine GHB are based on the oxidation of GHB to succinic semi-aldehyde (SSA), a reaction that occurs during metabolization *in vivo* *via* the enzyme GHB-dehydrogenase (GHB-DH).

**4.2.1 Colorimetric enzymatic assays**

Bravo *et al.* (2004) [80] developed a solution-endpoint- and a dipstick-assay for the determination of GHB in human urine. The identification was possible by coupling the oxidation reaction of GHB, *via* a cloned and isolated GHB-DH, to a reduction reaction of a tetrazolium pro-dye, resulting in the formation of a colored product (absorbance at 450 nm). Although these tests are easy to perform, providing enough sensitivity remains a critical issue, only ensuring 100 % true positives when a minimum of 100 µg/ml of GHB is present in urine.

Another test strip, commercially available by Drugcheck®, can detect GHB in human urine with a cut-off level of 10 µg/ml. Results are obtained within 10 min and a colour chart on the test strip has to be used for interpretation, next to a test strip for vitamin C, this compound showing cross-reactivity with the GHB test. Although this GHB test strip is more sensitive, detecting lower GHB concentrations, only a preliminary result is provided, without indication of the degree of intoxication [81].

**4.2.2 Enzymatic kit**

It has become clear from the tests mentioned above that there was an urgent need for a rapid and simple screening method to detect GHB in urine and serum samples in a more sensitive and semi-quantitative way. To this end, an enzymatic kit was commercialized in 2009 [77]. This kit also utilizes a recombinant GHB-DH to oxidize GHB to SSA, while the co-factor nicotinamide adenine dinucleotide (NAD+) is simultaneously reduced to NADH + H+, which absorbs at 340 nm. The test is adaptable to common clinical chemistry analyzers and requires only 10 µl of sample. Quantification is performed using 2 calibrators and 2 quality controls provided by the manufacturer, with a working range from 5 to 250 µg/ml. Results are obtained in about 10 minutes and interferences as well as cross-reactivities have been evaluated. A 4 % interference of GBL has been observed, which is stated to have no relevant implication since GBL is rapidly converted to GHB once ingested. Also per 1.06 g/L ethanol, a 3.0 µg/ml linear increase of false-positive GHB concentration was observed, so GHB concentrations of 8-20 µg/ml need careful interpretation, especially since GHB is commonly ingested with alcoholic beverages [11]. A cut-off level of 10 µg/ml for serum and 15 µg/ml for urine has been proposed [82,83].

Grenier *et al.* (2012) [84] evaluated the use of this enzymatic assay as a screening method in forensic matrices including whole blood and vitreous humour. When correlating the results of a variety of cases (sexual assaults, impaired drivers and deaths) with a GC-MS reference method, no false negatives and few false positives were observed, with post-mortem samples appearing to be more prone to testing false positive than ante-mortem samples. Although whole blood required protein precipitation with acetonitrile before analysis, analyst time savings can still be substantial compared to chromatography-based procedures. In addition, although very efficient GC-MS and LC-MS/MS procedures have been developed for GHB, integration with a battery of other tests on automated analyzers makes this assay valuable for *(clinical)* toxicology labs. However, Grenier *et al*. (2012) [84] found that a limitation of this test is that it may not be applicable to alternative matrices such as e.g. vitreous humour due to the observed high rate of false positives.

In summary, this test may be valuable for screening urine and serum samples in an emergency setting, for forensic applications and for other screening purposes [83].

*4.3 Other screening techniques*

 1H nuclear magnetic resonance (NMR) spectrometry has been used to detect GHB in urine and serum [85], as well as in oral fluid (600 µl) [75]. This technique is non-destructive and has little or no sample preparation requirements, and is therefore less labour-intensive than other techniques. Similarly, ion mobility spectrometry (IMS) showed promise as a screening method for GHB and related compounds present in urine samples [86]. Via direct injection using a split/splitless injection port and thermal desorption, the sample was brought directly into the IMS configuration without chromatographic separation, reducing analysis time and resulting in an estimated detection limit of 3 µg/ml.

In addition, CZE with indirect ultraviolet (UV) detection is capable of detecting high concentrations of GHB in urine samples following a simple 1:4 dilution with water. Calibration curves ranged from 80 to 1280 µg/ml [87]. For detection, indirect UV absorption using a chromophore in an electrolyte solution was necessary because the native molecule GHB has poor UV absorption [88,89]. Small adaptations of analytical conditions (co-ion, pH, etc.) further improved method sensitivity and selectivity and enabled the analysis of not only urine but also serum samples following 1:8 dilution with 3 mM NaOH, completely converting GBL to GHB (calibration curve ranged from 25 to 500 µg/ml) [90]. Although accurate and precise results may be obtained using CZE, the LLOQ is relatively high (ranging from 25 to 80 µg/ml and 5 to 60 µg/ml, dependent on urine density), when compared with chromatographic techniques (LLOQ ranging from 0.1 to 8 µg/ml). Therefore, these CZE-based methods are considered to be more suitable as an alternative screening method for a GHB overdose, being rapid and simple, rather than as a secondary confirmatory method.

*4.4 Chromatographic screening techniques*

When compared to colorimetric and enzymatic assays, chromatographic assays typically require more intensive and time-consuming sample preparation such as derivatization or conversion to GBL (see below). For example, Lebeau *et al.* (2000) [8] opted for a gas chromatography - flame ionization detection (GC-FID) screening method using headspace (HS) as injection technique following conversion of GHB to GBL, while confirmation of GHB (as GBL) was done by GC-MS. Also, in clinical practice, where the aim is to define a medical diagnosis and start a treatment, a non-specific detection such as GC-FID is sufficient, as stated by Blanchet *et al.* (2002) [42]. These authors determined GHB following derivatization with BF3-butanol.

Similarly, urinary organic acid assays based on silylation and GC-MS, more readily available than GHB assays in hospital laboratories, were investigated for their use to detect GHB in urine samples. However, if these methods included acidification of the samples during sample treatment, which favours conversion of GHB to GBL, only a small peak of GHB was visible, as can be expected [91]. In addition, silylated urea may elute closely to/co-elute with silylated GHB, having in addition similar MS properties. Therefore, it may be important to eliminate the urea interference by adding an urease treatment step to the sample preparation procedure, enabling the identification of GHB with higher confidence [92-94].

In addition, chromatographic methods used to screen for various compounds including GHB have been reported. Rasanen *et al.* (2010) [95] developed a headspace in-tube extraction GC-MS method to screen for hydroxylic methyl-derivatized organic acids, including GHB, in urine and extracted whole blood samples. In addition, a GC-MS method for the simultaneous screening in urine of 128 date-rape drugs, including GHB, 1,4-BD and GBL (using silylation), has been reported by Adamowicz and Kala (2010) [96].

Recently, an LC-MS/MS method has been reported to screen for elevated GHB concentrations in DBS obtained from newborns, to diagnose SSADH deficiency, a rare inherited metabolic disorder where GHB concentrations are increased because of a deficiency of the succinic semi-aldehyde dehydrogenase enzyme, responsible for conversion of SSA to succinate [68]. Although not intended for toxicology purposes, this methodology may also be applicable to screen DBS for exogenous GHB [97,98].

Next to these screening methods, several authors have reported simplified and rapid procedures to determine GHB with high-throughput, leading to the possibility of using actual confirmation methods also as a screening tool. Here, we mention only examples of these methods in which sample preparation is reduced or minimal. Details can be found in the next section and in Table 1. For example, Van hee *et al.* (2004) [36] determined GHB (and glycols) in low volume plasma and urine samples (20 µl) using GC-MS, by adding an excess silylation reagent directly to the biological sample. This procedure was recently modified by Meyer *et al.* (2010) [35], utilizing micro-wave assisted derivatization, another approach particularly useful in hospital laboratories of emergency departments, as quantitative results for urine samples can be obtained within 30 min using one-point calibration. Other examples of procedures with minimal hands-on time are those where derivatization reagents are applied directly “on spot”(in the case of DBS) or “in-vial” (in the case of HS-sampling) [41,44,67]. More recently, a multi-analyte ultra high performance LC-MS/MS (UHPLC-MS/MS) method has been reported, which may also be useful as a screening tool because of the easy sample preparation and resulting high-throughput [99].

5. Confirmation methods for clinical and forensic cases

Methods suitable for the confirmation of a presumed GHB-positive sample have preferably an LLOQ below or at the proposed cut-off level, should be selective for GHB and, if they deliver quantitative results, these should be reliable and accurate. Since it may be necessary to confirm the presence of GHB in more complex biological matrices and because more sophisticated chromatographic techniques are used, sample preparation becomes more important. Sample work-up is mostly more complicated than that used for colorimetric or enzymatic methods, which are primarily suited for urine and serum. Below, an overview of commonly used sample preparation procedures is given, followed by an overview of the used analytical techniques to separate and detect GHB (and analogues). Table 1 provides an overview of the different published procedures. To evaluate if a given method allows differentiation between exo- and endogenous GHB, the calibration range with the quantification limit is included. Also the choice of internal standard may influence the data quality and has therefore also been mentioned in the table [100]. As shown in the table, several compounds showing similarity with GHB have been used as internal standard. In MS-based methods, the use of a deuterated internal standard is recommended to compensate for variations during sample preparation, as well as during analysis. The deuterated form of GHB, GHB-d6, has been used widely for this purpose; a C-labelled internal standard is not commercially available (yet).

*5.1 Sample preparation*

The following techniques have been applied to treat biofluids, either alone but mostly combined: dilution, filtration, deproteinization, chemical modification, liquid-liquid extraction (LLE), solid-phase extraction (SPE), and HS extraction. These sample preparation procedures are often regarded as time-consuming and there has been a tendency to reduce manual sample handling by introducing new, fully automated techniques. It should be mentioned that the latter implies longer method development times and new skill requirements and may not always be implementable in smaller laboratories [101]. Furthermore, starting from the more traditional procedures, simplified extractionless procedures have been proposed such as dilution and direct derivatization (“on spot” and “in-vial”), together with micro-wave assisted derivatization and on-line derivatization techniques such as injection port derivatization. Some of these simplifications have been made possible due to improved separation and detection techniques such as tandem MS, resulting in procedures with minimal hands-on time. In addition, initial sample volume required for analysis may be reduced without loss of method sensitivity. The latter also depends on GHB recovery, a parameter worthwhile evaluating during optimization of sample treatment. Recovery should generally be reproducible and sufficient in terms of method sensitivity. Therefore, examples of strategies influencing recovery are also mentioned below.

**5.1.1 Dilution and filtration of the biological fluid**

Using appropriate separation and detection techniques, simple dilution of urine and serum samples, with or without subsequent filtration, may be sufficient as sample preparation [34,99,102]. This has been demonstrated by several LC-MS/MS methods, capable of quantifying GHB with sufficient sensitivity in these matrices. In addition, possible extraction difficulties arising from the hydrophilic nature of GHB are avoided. For example, urine has been diluted 1:20 [34] and 1:1 [99] with water, and 1:10 with acidic 10 % MeOH [102] prior to LC-MS/MS analysis. Alternatively, urine and serum samples have been diluted 1:4 with a buffer solution prior to CZE analysis with contactless conductivity detection (CZE-C4D) [103].

Important to note is that sufficient sensitivity has been obtained, as illustrated by Wood *et al*. (2004) [34], who compared method sensitivity (reflected in signal-to-noise (S/N)) of two sample pretreatment procedures prior to LC-MS/MS analysis. The first of these procedures consisted of a 1:20 dilution of urine samples (with deionised water containing internal standard); the second sample clean-up was based on a more time-demanding SPE extraction (OASIS® cartridges). Although an approximately 2-fold increase in sensitivity was observed when using the SPE cartridges, the authors were still able to use the simpler dilution method as it readily enabled measurement of endogenous GHB levels.

Although this dilute-and-shoot approach is simple and convenient, with minimal hands-on time, assessment of matrix effects is strongly advised, as in any LC-MS/MS-based procedure, because matrix components may strongly influence the ionization of GHB in the MS source. To compensate for any effect owing to the matrix, a stable isotopically-labelled internal standard should be included in the procedure [34]. Assuming a similar degree of alteration of the response of this internal standard, matrix effects can be compensated for.

In addition, samples have been diluted to reduce the influence of the original matrix during sample treatment and analysis. For example, the effect of the matrix during solid-phase micro-extraction (SPME) may be reduced by diluting the original sample [38,104]. Similarly, samples were diluted with water before LLE [105,106] or SPE [99,107-109]. For example, Elian *et al*. (2011) [107] assumed that a 50-µl sample and synthetic urine, consisting of inorganic salts and proteins in an aqueous medium, would act in a similar way as 50 µl deionized water, if these were all diluted in 4 ml of water prior to SPE. As a consequence, calibrators and controls could be prepared in water instead of blank matrix, which is especially of interest for GHB, endogenously present in human samples.

**5.1.2 Deproteinization of the biological fluid**

For compounds with low protein binding such as GHB, protein precipitation is an adequate and easy technique to remove a variety of interferences present in blood and plasma, such as blood cells, proteins and lipids prior to analysis [32,99]. To illustrate, Shima *et al*. (2005) [32] compared protein precipitation with various SPE and LLE techniques to clean up urine samples, and found that protein precipitation led to the highest GHB recovery and cleanest chromatograms. Generally, there are 4 protein precipitation techniques: organic solvents, acids, salts and metal ions [110]. To our knowledge, for GHB analysis, only the first 3 have been applied.

As organic solvent, acetonitrile [e.g. 16,35,37,111-114], methanol (MeOH) [32,115] or a combination of both (ice-cold acetonitrile:MeOH 85:15 v:v [99,109]) and acetone [31,116] have been used. Also water:MeOH (3:97, v:v) has been used [117]. Placing samples in a freezer for at least 10 min before centrifugation may help to obtain complete precipitation [99].

Organic solvents and acids have also been used simultaneously to improve protein precipitation and, in addition, GHB recovery. For example, the addition of sulphuric acid during protein precipitation using acetonitrile was found to increase the recovery of GHB from 50 to 90 % [5,33,66]. Similarly, acidified methanol has been used for whole blood protein precipitation [102].

Cold perchloric acid [27,29,54] has been added alone to plasma samples, thereby combining deproteinization and lactone-formation (see below). Finally, anhydrous sodium sulphate (Na2SO4) has been added to biological fluids prior to HS-trap analysis for protein precipitation and for salting-out purposes [41].

Similar to the above mentioned dilute-and-shoot approach, it is important to evaluate matrix effects, particularly when protein precipitation is (almost) the only sample preparation technique prior to LC-MS/MS analysis. For example, when blood or serum samples were subjected to protein precipitation, with subsequent centrifugation and 1:1 dilution of the supernatant with acidified water prior to LC-MS/MS analysis, about 40 % suppression of the GHB signal was observed. Again, the use of a deuterated internal standard such as GHB-d6 is emphasized, since it is able to compensate for the matrix effect [102]. To our knowledge, only three sample preparation procedures merely consist of protein precipitation prior to LC-MS/MS injection, with only two evaluating matrix effects [113,114,117]. For example, one procedure that did evaluate matrix effects saw a 16 to 27 % enhancement of GHB ionization which was compensated for by the use of GHB-d6 [117].

**5.1.3 Chemical modification of GHB**

Because GHB is a polar (sometimes anionic) molecule, with lactone-formation seen at high injector-port temperatures or induced chemically (at low pH), chemical modification prior to GC is necessary for reliable quantification [75,118]. For GC analysis, two major strategies have been applied: either lactone-formation through the addition of acid, or derivatization using various derivatization reagents. Formation of a derivative prior to LC analysis and detection has proven to be useful as well; however, it has been applied to a lesser extent when compared to its use in GC-based applications. Both strategies – lactone formation and derivatization – may improve extraction properties of GHB and/or chromatographic analysis, as discussed more into detail below. Fig. 2 gives a schematic overview of possible derivatization procedures, with resulting derivatives of GHB, using common derivatization reagents.

*5.1.3.1 Chemical modification to improve extraction*

GHB undergoes intra-molecular esterification within minutes in acidic environment [40] and the GBL formed is more easily extracted from biological matrices than GHB. Lactone-formation has been accomplished by the addition of 6 N hydrochloric acid (urine samples [27,29]; [119]), concentrated sulphuric acid [8,120], 20 % trifluoroacetic acid [121] or 1.6 or 0.8 N perchloric acid (plasma [27,29]) to plasma, urine or oral fluid samples. By adding perchloric acid to plasma samples, lactone-formation and deproteinization were performed in a single step. Subsequently, the acidified sample or the supernatant following centrifugation [27,29] were placed at room temperature [119,120] for 5 min [8], at 80 °C for 20 min [27,29] or at 75 °C for 1 h [121] to allow for complete conversion. As a consequence, differentiation between the initial GBL present in the sample and the GBL formed as a result of the acid-induced cyclization of GHB is no longer possible [105], unless two aliquots of the same sample are analyzed, one with and one without acid treatment [8,29].

In addition, derivatization reagents suitable for “in-situ” or “in-vial” derivatization can be added directly to the sample matrix, thereby allowing the samples to be analyzed by solid-phase micro extraction (SPME) or HS-based extraction (and injection) techniques by forming a more volatile derivative of GHB. Hexylchloroformate, in the presence of the catalyst pyridine, has been used to derivatize the carboxylic group of GHB [38,104], while dimethylsulphate (DMS) in alkaline medium has been utilized to modify both functional groups [41,95].

To overcome the difficulties seen when extracting the hydrophilic and small analyte GHB in those methods requiring derivatization, extractionless derivatization procedures have been reported. In addition to an expected improvement in recovery, sample preparation time, as well as organic solvent waste is reduced. Van hee *et al*. (2004) [36] were the first to report on an extractionless sample preparation, based on the direct derivatization of GHB in biofluids with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The addition of excess derivatization reagent to a 20 µl sample of biofluid (serum, plasma, urine) resulted in a simple and fast method with sufficient sensitivity for routine toxicological analysis. Similarly, starting from 1 µl oral fluid, an extractionless procedure with direct derivatization has been reported, allowing determination of exogenous GHB concentrations [69]. Furthermore, GHB has been derivatized directly (“on spot”) in DBS witha mixture of trifluoroacetic acid (TFAA) and heptafluorobutanol (HFB-OH), thereby omitting the extraction step [44,67].

*5.1.3.2 Chemical modification to improve chromatographic analysis and detection*

Apart from improving or facilitating extraction, chemical modification may also improve chromatographic analysis and detection. GC properties of GHB are improved by conversion to its more volatile and stable lactone-form GBL, achieved by applying the same procedures as to improve extraction *via* GBL formation (see above). Secondly, various derivatization reagents have been used to increase its molecular weight, at the same time decreasing its polarity, thereby enhancing volatility, separation efficiency and/or selectivity, and consequently, method sensitivity.

As shown in Table 1, silylation is widely used to derivatize GHB off-line in GC-based applications. Mainly BSTFA [e.g. 5,16,31-33,35,36,43,49,50,59,60,62,66,69,91,111,112,122-125,127] has been applied, next to N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) [105,115,126] and N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA) [37,106]. Using these reagents, the hydroxyl and carboxyl functional groups of GHB are derivatized simultaneously, thereby avoiding lactone-formation since no acidic conditions are used [32]. Moreover, to avoid GBL formation and GHB losses during evaporation, Kimura *et al.* (2003) [91] alkalinized urine samples prior to derivatization, producing the non-volatile salt form of GHB. Furthermore, the resulting di-trimethylsilylderivative of GHB (Fig. 2) can be injected directly into the GC-MS, without removal of excess reagent. Of course, the latter requires increased maintenance of the injection port and MS source to prevent contamination between runs. Another issue is the possibility of co-eluting di-TMS urea, requiring baseline separation of GHB and the urea di-TMS derivative under the GC conditions used. As silylating reactions mostly require heating for 5 to 30 min, injection-port [106] and micro-wave assisted silylation [35] may offer valuable alternatives to reduce technical time. Also the aforementioned derivatization reactions improve chromatographic analysis and detection.

Although derivatization is primarily known for its use in GC applications, it may also be applied in LC-based separations. For example, to allow fluorescence detection, the carboxylic group has been derivatized by adding 4-bromomethyl-7-methoxy coumarin (Br-MMC) to an aqueous-free sample residue in the presence of dibenzo-18-crown-6-ether acting as a catalyst to improve the reaction yield [89]. Furthermore, butylation of the carboxylic function of GHB using HCl n-butanol improved detection with ion-trap mass spectrometry [128].

**5.1.4 Liquid-liquid extraction (LLE) of GHB or GBL**

 *5.1.4.1 Liquid-liquid extraction of GHB*

Solvents commonly used to extract GHB from biological fluids include ethylacetate [49,50,59,60,62,106,122-128], t-butylmethylether [105,128] and hexane [43]. Because the physical properties of GHB make it a poor candidate for LLE, various approaches to enhance the transfer of GHB to the organic solvent (hence increasing its recovery) have been reported. GHB has to be in its uncharged or neutral form to obtain an optimum extraction yield and selectivity, influenced by the choice of solvent, pH and additives [101]. Therefore, the charge of the carboxylic group (pKa 4.6-4.8) has been influenced by the addition of 0.1 M HCl or cold 0.1 N H2SO4 to urine, serum and blood samples, enhancing its transfer to ethylacetate [59,122,128]. Also, for whole blood samples, Pan *et al.* (2001) [37] reported the use of water scavenging material such as dimethoxypropane and N,N-dimethylformamide (80:20) to facilitate GHB extraction. Furthermore, salting-out approaches have been reported, whereby the ionic strength of the aqueous phase increases, improving the partitioning of relatively water-soluble analytes between two immiscible phases [105]. For this purpose, saturated salt solutions such as saturated ammoniumchloride buffer [51,125,127] have been added to the test tubes or NaCl (solid salt) has been pre-loaded [105] prior to extraction.

 *5.1.4.2 Liquid-liquid extraction of GBL*

Following lactone-formation (see above), GBL has been extracted from biologic fluids with methylenechloride [8], chloroform [119,121] or benzene [27], solvents that are preferentially avoided in the modern laboratory. Since GBL may be protonated under the acidic conditions required for complete conversion, recoveries can be improved by adding sodium chloride to the solution for salting-out purposes, but also by neutralizing (pH 6-7) the initial acidic pH (pH 1) using e.g. phosphate buffer and sodium hydroxide [27,121]. Following LLE, the mixture is generally centrifuged and the supernatant subsequently concentrated, but not completely evaporated since GBL may be lost during evaporation to dryness, being more volatile than the free acid [92]. As an example, it was found essential to evaporate with low nitrogen flow and at low temperatures (max 35 °C) to avoid unacceptable losses of GBL [27].

**5.1.5 Solid phase extraction (SPE) of GHB**

A first type of SPE sorbent used to extract GHB from biofluids is (strong) anion exchange. When using this type of cartridges, the classical SPE procedure of conditioning, loading, washing, drying and eluting has been followed. The interaction is based on ion exchange chemistry, whereby the organic moiety or quaternary ammonium material bonded to the solid matrix maintains its positive charge over the whole pH range, allowing pH-dependent interaction with GHB. At neutral pH, the carboxylic group will be negatively charged (pKa 4.6-4.8), and will interact with the positively charged sorbent. To elute GHB, it is necessary to neutralize its negative charge using an acidic elution solvent [107,108,129].

In addition, SPE cartridges can also be used to retain interfering substances, allowing the analyte of interest to pass through the sorbent and collecting the resulting eluate for further analysis. For this purpose, Clean Screen® SPE cartridges have been applied to clean-up vitreous humour, blood and urine. The collected eluate contained GHB without substances that could interfere during subsequent analysis [31,43,126]. In addition, a (strong) cation exchange sorbent can be used for sample clean-up of whole blood samples following protein precipitation. Introducing this additional clean-up resulted in improved peak shape of GHB and in reduced baseline noise [99,109].

An advantage of SPE is that it can be automated more easily than current precipitation or derivatization techniques which typically require off-line manual operations [107]. To illustrate this, an automated SPE (Oasis® HLB 30) using a 96-well plate has recently been applied for the extraction of GHB from whole blood samples, following protein precipitation [99]. Combining this automated SPE with LC-MS/MS resulted in a high-throughput method suitable for screening more than 6000 samples a year [99].

Also SPME, as a modification of the more classical SPE, has been introduced. In contrast to conventional extraction methods, which use multi-step techniques and excess organic solvents, SPME only consists of one solvent-free step to concentrate the analytes of interest. This technique has been applied to determine GHB (derivatized with hexylchloroformate) in urine samples, using a fused silica fibre coated with a stationary phase adsorbing the analytes of interest. The SPME fiber can be brought directly in the sample, or alternatively, in the headspace (see further, headspace extraction of GHB) [38,104].

**5.1.6 Headspace extraction of GBL or derivatized GHB**

Only a minority of the presented GC methods uses HS as extraction and injection technique. The reason for this may lie not only in the more complex optimization of these procedures but also in the fact that typically a larger sample volume is required to obtain similar sensitivity as compared to more traditional sample preparation procedures such as LLE or SPE [29,104]. Also the requirement for a more specific configuration, which also may imply the use of a more specific analytical column (see Table 1) limits its general use. Nonetheless, these techniques have the advantage that GHB, in a derivatized form or as GBL, can be extracted directly from the aqueous sample, requiring less manual operations, being fully automatable, consuming less solvent (being solvent-free) and saving technical time [38,104]. Sample preparation is mostly limited to adding the following to a HS vial: an aliquot of the sample, anhydrous salt to enhance the transfer of the analyte of interest to the headspace (increasing its recovery), derivatization reagents or acid for lactone-formation. Next, after proper sealing of the vial, it can be placed in the HS oven for analysis.

 *5.1.6.1 Headspace extraction of derivatized GHB*

Combining “in-vial” derivatization with headspace injection techniques may extend the application range normally reserved for volatile compounds to semi- or non-volatile analytes such as GHB. Following derivatization with hexylchloroformate or dimethylsulphate, derivatized GHB has been extracted using SPME or HS-trap, respectively [38,41,95]. Both methods have minimal sample preparation time. The method using SPME is one of the most sensitive methods reported, having an LLOQ of 0.1 µg/ml, starting from 0.5 ml urine.The HS-trap method is suited for the determination of GHB in various biological fluids, requiring only 100 µl of sample.

 *5.1.6.2 Headspace extraction of GBL*

A static HS method has been described for the determination of GHB, based on LLE of 1 ml urine or whole blood samples, followed by conversion to GBL [8]. Headspace SPME and solid-phase dynamic extraction (SPDE) have also been applied to determine GHB as total GBL in plasma and urine samples, resulting in methods with sufficient sensitivity (LLOQ from 1 - 5 µg/ml) but requiring relatively large sample volumes (ranging from 0.5 to 1.0 ml) compared to other sample preparation techniques (0.02 – 0.5 ml) [29,120].

*5.2 Chromatographic analysis and detection*

**5.2.1 Gas chromatography**

Although the nature of GHB does not favour the use of GC (see above), it remains the most popular separation technique of the last two decades, enabled by the use of appropriate sample preparation techniques. Toxicological analyses are commonly performed using an analytical column with a stationary phase consisting of silica with 95 % methyl - 5 % phenyl groups, which is also well suited for the determination of derivatized GHB and/or GBL (and analogues), reflected in its wide use. The majority of GC-based methods focus on the detection of GHB, either in derivatized form or in the form of GBL, while a few methods also include simultaneous analysis of glycols, BHB, GHV, 1,4-BD and/or SSA [31,35,36,39,123,124].

 *5.2.1.1. Gas chromatography – flame ionization detection*

Although various authors use this universal detector to initially screen for GHB, followed by confirmation using GC-MS [8,56], Jones *et al*. (2007) [116] used GC- flame ionization detection (FID) to quantify GHB as GBL in blood samples within a wide calibration range, starting at 8 µg/ml.

 *5.2.1.2 Gas chromatography – mass spectrometry*

To unequivocally demonstrate and determine GHB in biological fluids, GC is preferably used in conjunction with mass spectrometry. It has been used in electron impact ionization (EI) and positive or negative chemical ionization mode (PICI or NICI). For quantification, the MS operates in SIM (selected ion monitoring) mode, following those *m/z* ions typical for GBL or derivatized GHB. Derivatization using silylating or other derivatizing reagents is generally advantageous for MS detection, by increasing the molecular weight and the fragments’ masses. Hence, more selective ions are formed than those formed from GBL (*m/z* 42, 56 and 86 in EI mode). In addition, fragmentation of the di-TMS-derivative via CI instead of EI results in mass spectra with more abundant and higher molecular weight ions [59]. PICI has been used by Kerrigan (2002) [59] and Chen *et al.* (2003) [111] to quantify GHB in various biofluids following silylation and by Lenz *et al.* (2009) [120] and Frison *et al.* (2000) [29] following GHB conversion to GBL. Although one method had a relatively lower LLOQ of 0.4 µg/ml, no relevant gain in sensitivity has been observed when compared to GC methods where the MS performs in EI mode. On the other hand, using the MS in NICI mode to quantify GHB as GBL in plasma samples has been shown suitable for the determination of endogenous concentrations, with a calibration range situated in the low µg/ml range [119].

Although not routinely performed using GC-based methods, simultaneous analysis of GHB and 1,4-BD or other compounds such as BHB and SSA is possible, as was done by Lora-Tamayo *et al*. and Sakurada *et al.*, [123,124] respectively, who only slightly modified the method of Couper and Logan (2000) [122]. GHV and GHB can be analyzed simultaneously [31] and recently, Andresen-Streichert *et al*. (2013) [39] reported a GC-MS method for the simultaneous analysis of GHB and GHV in urine samples, with an extraction and derivatization procedure based on the method published by Kerrigan (2002) [59].

 *5.2.1.3 Gas chromatography - tandem mass spectrometry*

Coupling tandem MS to GC enables the monitoring of a selected transition from a parent ion to (a) specific daughter ion(s), which may reduce the requirement for time-consuming sample clean-up techniques. However, although very high sensitivity as a result of increased selectivity may be valuable for hair analysis, the advantage of being able to detect low GHB levels by MS/MS techniques is not crucial for blood and urine samples, since GHB is readily endogenously present at relatively high concentrations (sub- and low- microgram-per-milliliter range). Nonetheless, MS/MS still may offer improvements in peak shape required for reliable integration [33]. Although tandem MS may have the advantage over existing methodologies of providing spectra free from background contaminants and thus being more selective, it remains or becomes even more important to evaluate if the di-TMS derivative of GHB is free from interferences from compounds with the same precursor ion (*m/z* 233), such as its positional isomers, AHB and BHB [33].

**5.2.2 Liquid chromatography**

The minority of confirmatory methods uses LC to determine GHB in biofluids. This may be due to historical reasons, since GC has been longer and more widely available for routine analyses in toxicological laboratories, but also because of practical reasons, since poor retention of the native molecule on classical reversed phase (RP) columns is expected. Nonetheless, LC techniques may offer advantages over existing GC methods. For one, although similar sensitivity has been obtained, workload and use of toxic solvents may be reduced, since the introduction of tandem MS has resulted in simpler sample preparations, such as dilute-and-shoot, without the requirement for derivatization or conversion prior to analysis. The fact that no conversion is required makes that several LC methods can detect GHB and its precursor GBL simultaneously, while most of the reported GC methods require additional analyses [99]. Finally, introduction of ultra-high performance LC (UHPLC), having higher efficiency than traditional high performance LC (HPLC), and automated sample preparation techniques have further led to the development of highly useful high-throughput LC-MS/MS methods [34,68,99,102].

*5.2.2.1 Liquid chromatography – ultraviolet detection/ fluorescence detection*

Since GHB has no chromophoric group, UV-detection is only possible at a low wavelength (220 nm), as reported by De Vriendt *et al.* (2001) [108]. Starting from 60-µl plasma samples, quantification was possible in a range from 10 to 750 µg/ml, the LLOQ being 5 to 10-fold higher than the majority of confirmatory methods reported here. Introducing an UV-active or fluorescent group through derivatization should allow for enhanced sensitivity and improved certainty of identification, as illustrated by Zacharis *et al.* (2004) [89]. These authors derivatized GHB, producing a highly fluorescent derivative starting from 500-µl oral fluid samples, with the lowest calibrator corresponding to 0.25 µg/ml.

*5.2.2.2 Liquid chromatography - tandem mass spectrometry*

UHPLC-MS/MS has the potential for shorter run times and improved sensitivity and precision compared to more traditional separation methods such as HPLC-UV or HPLC-Fluo, also facilitated by the possibility to use a stable isotopically labelled internal standard. To illustrate, Fung *et al*. (2004) [129] modified the LC-UV method described above [108] to a method suitable for LC-MS/MS, and although a slightly higher initial sample volume was required -100 instead of 60 µl - the run time was reduced to 5 min and sensitivity was increased 100-fold (LLOQ equal to 0.1 µg/ml).

Also, LC-MS/MS may allow for the simultaneous analysis of GHB and its precursors, GBL and 1,4-BD [34], using isocratic elution (with 10 % MeOH or acetonitrile) or a slightly rising gradient. Adequate baseline separation of not only GBL and 1,4-BD but also of AHB, BHB and GVL from GHB has been shown [34,68,102]. This baseline separation of GHB and its positional isomers is particularly important for adequate identification of GHB using one parent and one product ion. Moreover, since under some conditions in ESI(+), the molecule might lose water within the instrument source with the formation of GBL, it is of interest that the method can distinguish between in-source generated GBL or GHB-H2O+ and actual GBL in a sample [34,102,109]. Interesting to note is that one method [102] used this in-source conversion of GHB to GBL to obtain sufficient sensitivity for GHB determination in whole blood samples, while others [34,109] only observed a relatively low conversion (factor 6 %) unsuitable for GHB quantification.

Alternatively, to counter the detection of small *m/z* ions typical for GHB (*m/z* parent ion= 103), recently, an LC-MS/MS method for GHB in human serum has been reported, where quantification was based on the fragmentation of adducts formed with components of the mobile phase, more specifically on the fragmentation of the GHB sodium acetate adduct in ESI(-) (*m/z* 185) [117] .

Tandem MS has been used in both atmospheric pressure chemical ionization (APCI) and ESI mode, with ESI(+) producing only one product ion with significant abundance and ESI(-) revealing three abundant transition products. The latter is more beneficial for method sensitivity and selectivity [107,109]. On the other hand, reversed phase C18 columns frequently used for GHB separation require acidified mobile phases to better control the retention of GHB (being a weak acid with a pKa 4.6 it is only uncharged in acidic mobile phases) [99]. This may lead to a restriction to work in the ESI (+) mode, since the acidic conditions used may reduce the response of GHB in ESI(-) mode [99,109]. However, Forni *et al*. (2013) reported lower background noise under their chromatographic conditions with the MS/MS operating in ESI(-) as compared to ESI(+) [68].

Sørensen *et al.* (2012) [109] and Lott *et al*. (2012) [114] suggested the use of hydrophilic interaction liquid chromatography (HILIC) as an alternative to overcome this problem and to improve retention and chromatographic separation of small and polar molecules. HILIC allows for chromatography to be performed under neutral conditions, optimal for separation of GHB and analogues, which in addition also prevents inter-conversion between GHB and GBL [109]. To compare, using a typical C18 reversed phase column, GHB elutes first, followed by 1,4-BD and GBL, while using HILIC the elution order is reversed, which simplifies optimization of the retention time of GHB by adjusting the composition of the mobile phase [109]. Despite these advantages, to reduce analysis costs of high-troughput methods, one may opt not to use HILIC methods with acetonitrile, given its higher toxicity and price than e.g. MeOH. Furthermore, also practical reasons, such as instrumental back-up, may influence the choice to use RP-columns and -chromatographic separation rather than HILIC-based chromatography [99].

*5.3 Non-chromatographic techniques*

Gong *et al*. (2008) [103] reported a CZE-C4D method to determine GHB in urine and serum samples. Although not commonly used for toxicological purposes, this technique is well-suited for the determination of small and ionic molecules such as GHB (anionic form) [118]. Separation and detection of AHB, BHB and GHB was achieved without preceding extraction or derivatization but only by a simple 1:4 dilution with an optimized separation buffer with a pH > 4.7 to deprotonate the analytes and to obtain them in anionic form. In addition, the more alkaline pH also inhibits conversion of GHB to GBL. The method showed sufficient sensitivity to discriminate between endo- and exogenous GHB levels in urine samples (cut-off 10 µg/ml). Also, instrumentation is less expensive than other instruments used in clinical and forensic laboratories and a portable instrument may allow for on-site analysis of urine samples from suspected GHB-intoxications [103].

6. Conclusion

To conclude, various screening and confirmation methods are available to determine GHB (and analogues if required) in biological fluids. GHB screening/analysis in a toxicological laboratory is mostly performed based on a suspected ingestion of this club drug, supported by information of the police or physician, rather than on a routine basis, as is the case for more widely abused drugs such as cannabinoids, amphetamines and opioids [54,116]. However, routine screening has become possible, not only by the commercialization of an enzymatic kit for GHB applicable on common chemical analyzers, but also by the availability of more simplified GC-MS methods and more sophisticated techniques such as UHPLC-MS/MS, which - when combined with automated sample preparation procedures - allow high-throughput. To confirm the presence of GHB in biological fluids, GC has remained the most widely used separation technique during the last twenty years, despite the small and polar nature of GHB, requiring conversion to GBL or derivatization to a more volatile and stable form. However, LC-based applications coupled to tandem MS are increasingly gaining interest as they may offer the advantage of more simple sample preparation techniques (e.g. no derivatization) or dilute-and-shoot. Of course, when sample preparation is minimal, matrix effects require special consideration.Furthermore, despite the advantages of reduced workload and shortened analysis time that tandem MS-techniques may offer, baseline separation of GHB from GBL and from its isomers AHB and BHB, achieved by adequate chromatography, remains important to avoid interference (respectively by in-source formation of GBL during MS/MS analysis and by similar transitions) [33]. As to method sensitivity, both GC- and LC-based applications offer similar LLOQ’s, but as Kankaanpää *et al*. (2007) has nicely pointed out “the challenge is not to reach as low GHB concentration levels as possible, but to interpret the results correctly being able to make a distinction between use of GHB and endogenous levels” [105]. Indeed, once a result has been obtained using the above mentioned screening and confirmation methods, the interpretation is a second challenge for the toxicologist, whereby the analysis of different matrices may be useful for correct interpretation. For example, Kintz *et al.* (2005) [52] suggested the analysis of multiple specimens including different types of blood and vitreous humour in the case of a suspected GHB-related death. On the other hand, the question arises whether or not the analysis of alternative matrices such as oral fluid, sweat and hair has the potential to extend the detection window and/or to bring added-value to results obtained from analysis of the more conventional matrices. Furthermore, the newly reported metabolite GHB-glucuronide [74] may be a promising new biomarker of GHB exposure, although additional research is required.

**Table 1** Overview of confirmation methods to determine GHB in biological fluids, in alphabetic order and sorted by analytical technique (GC, LC and other)

**GC**

*Unless specified: GC-MS: 1 µl injected in splitless injection, MS EI ionization mode and helium as carrier gas; \*If method includes conversion of GHB to GBL: without acidification of the sample: determination of original GBL concentration possible*

| Ref. | Analyte | Matrix (sample volume) | Sample preparation | Stationary phase(total run time)  | IS GHB | Calibration Range GHB | Remarks  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Abanades et al., 2006 [5];Abanades et al., 2007 [66] | GHB | UrinePlasmaOral fluid(100 µl)(Sweat) | **PP**: 150 µl acetonitrile +50 µl 0.1 M sulphuric acid **Derivatization**: 50 µl (BSTFA + 1% TMCS) 70 °C 30 min | 5 % phenyl – 95 % methylpolysiloxane12 m x 0.2 mm, 0.33 µm(14 min) | GHB-d6 | 0.2-300 µg/ml |  |
| Andresen et al., 2010[50] | GHB | UrineSerum(100 µl) | **LLE**: 200 µl 0.1 M HCl + 1 ml ethylacetate **Derivatization**: 100 µl ethylacetate + 50 µl (BSTFA + 1% TMCS)  | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm(22.7 min) | GHB-d6 | 2-12 µg/ml |  |
| Andresen-Streichert et al., 2013 [39] | GHBGHV | Urine (100 µl) | **LLE**: 200 µl 0.1 M HCl + 1 ml ethylacetate**Derivatization**: 50 µl acetonitrile + 25 µl (BSTFA + 1% TMCS) | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm(16.22 min) | GHB-d6 | (NR for GHB) |  |
| Blair et al., 2000[104] | GHB | Urine(0.5 ml) | **Derivatization**:40 µl pyridine + 24 µl hexylchloroformate 40 °C 5 min**Solution SPME**: + 2ml deionized water + 1 ml pH 7 buffer; PDMS SPME fiber 12 min 40 °C | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm(23.33 min) | GHB-d6 | 5-500 µg/ml  | SPME GC-Q-trap |
| Bosman and Lusthof, 2004[54] | GHB\* | Urine Blood (1 ml) | **PP** **+ Conversion of GHB to GBL**:1 ml 1 M perchloric acid1 ml supernatant: 80 °C 20 min**LLE** **of GBL**: 300 mg NaCl, pH 6.5 (1 ml 1.5 N phosphate buffer + 350 µl 2.5 M NaOH), 5 ml chloroform | 100 % polydimethylsiloxane12 m x 0.2 mm, 0.33 µm (15 min) | GHB-d6 | 5-40 µg/ml | Case reportsSplit injection (10:1) |
| Brenneisen et al., 2004[121] | GHB\* | Urine (2 ml)Plasma (0.5 ml)Oral fluid (1 ml) | **Conversion of GHB to GBL**: 0.5 ml 20 % trifluoroactetic acid, 75 °C 1 h**LLE of GBL**: 0.55 mL 2 M NaOH (pH adjusting to 6.5) + 3 ml chloroform | 5 % phenyl – 95 % methylpolysiloxane25 m x 0.2 mm, 0.33 µm(10.1 min) | GVL | 10-50 µg/ml | 4 µl injected |
| Brown et al., 2007[38] | GHBMAMP MDMAKET | Urine(1 ml) | **Derivatization**: 10 µl hexylchloroformate + 40 µl pyridine**Headspace SPME of derivatized GHB**: 0.5 ml derivatized sample+ 1 ml water; 100 µm PDMS fiber 90 °C 20 min; 1 min desorption at 225 °C | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.10 µm (12 min) | GHB-d6 | 0.1-20 µg/ml | SPME  |
| Chen et al., 2003[111] | GHB | Plasma(100 µl) | **PP**: 2ml acetonitrile 50 µl supernatant evaporated**Derivatization**: 100 µl BSTFA 75 °C 15 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 1 µm(9 min) | GHB-d6 | 2.5-250 µg/ml | GC-PCI-MS(ammonia as reagent gas) |
| Couper and Logan, 2000[121] | GHB | UrinePlasma(1 ml) | **LLE**: 250 µl cold 0.1N sulphuric acid + 6 ml ethylacetate (2 x)**Derivatization**: 30 µl (BSTFA + 1% TMCS) + 60 µl acetonitrile70°C 15 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.33 µm (14 min) | diethyleneglycol | 1-200 µg/ml urine1-100 µg/ml plasma | 2 µl injected |
| Crookes et al., 2004[62] | GHB | Urine(2 ml) | **LLE**: + 0.36 M sulphuric acid to obtain pH 2.75; + 3 ml ethylacetate**Derivatization**: 100 µl (BSTFA + 1% TMCS) 60°C 30 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm(26.2 min) | GHB-d6 | 0.2-5 µg/ml | 2 µl injected |
| De Paoli and Bell, 2008[69] | GHB | Oral fluid(1 µl) | **Derivatization**: 97 µl (BSTFA + 1 % TMCS) 50 °C 30 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.5 µm(16 min) | GHB-d6 | 0.5-50 µg/ml | Split injection (10:1) |
| Duer et al., 2001[115] | GHB | UrineBloodVitreous humour(25 µl) | **PP**: 1.225 ml GHV methanolic solution**Derivatization**: 100 µl MSTFA + 100 µl ethylactetate 60 °C 30 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (24.7 min) | GHVGHB-d6 | Standard addition(10-500 µg/ml) | *GBL determination:* conversion to GHB in alkaline conditions (using 1 ml 10mM NaOH in methanol, 1 h 60 °C) following GHB analysis; *1,4-BD determinatio*n: di(dinitrobenzoyl) derivative using HPLC-UV |
| Elian, 2000[125] | GHB | Urine(50 µl) | **LLE**: 50 µl IS + 0.5 ml saturated ammonium chloride buffer + 3 ml ethylacetate **Derivatization**: 50 µl (BSTFA + 1% TMCS) + 50 µl ethylacetate 30 min RT  | 100 % polydimethylsiloxane12 m x 0.2 mm, 0.33 µm (9.7 min)  | GHB-d6 | 2-50 µg/ml | 2 µl injected |
| Elian, 2001[127] | GHB | Blood (50 µl) | **LLE**: 0.5 ml saturated ammonium chloride buffer + 3 ml ethylacetate **Derivatization**: 20 µl (BSTFA + 1% TMCS) + 80 µl ethylacetate 70 °C 20 min | 100 % polydimethylsiloxane12m x 0.2mm, 0.33µm(11.0 min) | GHB-d6 | 1-200 µg/ml | 2 µl injected |
| Elie et al., 2012[106] | GHB | Urine(300 µl) | **LLE** : 300 µl urine + 900 µl ethylacetate **Derivatization** : 50 µl acetonitrile + 50 µl (MTBSTFA+1%TBCS) | 100 % polydimethylsiloxane30 m x 0.25 mm, 0.25 µm(15 min) | *Trans*-4-hydroxycrotonic acid | 0.17-1.67 µg/ml | Injection port silylation: split/splitless injector with programmable pneumatic control  |
| Elliott, 2004 [56] | GHB  | UrinePlasma(1 ml) | **LLE**: 250 µl 0.05 M sulphuric acid + 6.0 ml ethylacetate **Derivatization**: 75 µl (BSTFA + 1% TMCS) 90 °C 5 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (9 min) | GHB-d6 | 5-200 µg/ml | 1) GC-FID screening method for GHB identification *via* GBL conversion2) GC-MS determination *via* derivatization (presented in detail) |
| Elliott, 2004 [45];Elliott et al., 2004 [60] | GHB | UrineBloodVitreous humour(100 µl) | **LLE**: 50 µl cold 0.05 M sulphuric acid + 0.5 ml ethylacetate **Derivatization**: 75 µl (BSTFA + 1% TMCS) 90 °C 5 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (9 min) | diethyleneglycolGHB-d6 | 6.25-100 µg/ml (urine)1-100 µg/ml (plasma) | Comparison with GC-FID method [25] |
| Ferrara et al., 1993[27] | GHB\* | UrinePlasma (2 ml) | **Conversion GHB to GBL**: 2 ml cold 0.8 N perchloric acid, supernatant (plasma **PP**) 0.2 ml 6 N HCl (urine) 80 ° C 20 min**LLE of GBL**: 300 mg NaCl, 1 ml pH 6.5 (1 ml 1.5 N phosphate buffer + 5 N NaOH), 8 ml (plasma) or 6 ml (urine) benzene | 100 % polydimethylsiloxane12 m x 0.2 mm, 0.33 µm (15.6 min) | δ-valerolactone | 2-150 µg/ml urine2-200 µg/ml plasma |  |
| Frison et al., 2000[29] | GHB\* | UrinePlasma(0.5 ml) | **Conversion GHB to GBL**: 100 µl cold 1.6 N perchloric acid, supernatant (plasma **PP**)25 µl 6 N HCl (urine) 80 ° C 20 min**Headspace SPME of GBL**: 500 mg solid phosphate buffer 50-µm CW/TPR SPME fiber10 min 70 °C | acid-modified polyethylene glycol phase25 m x 0.2 mm, 0.3 µm(9.8 min)  | GBL-d6 | 5-150 µg/ml urine1-100 µg/ml plasma | SPME GC-PCI-MS (methane as reagent gas) |
| Fukui et al., 2003[119] | GHB\* | Plasma (200 µl) | **Conversion GHB to GBL**: 0.5 ml 6 M HCl**LLE of GBL**: 2 ml dichloromethane (2 x)evaporation to 100 µl  | polyethyleneglycol30 m x 0.32 mm, 0.25 µm(11.5 min) | GBL-d6 | 0.01-1 µg/ml  | GC-NCI-MS |
| Ingels et al., 2010[44]; Ingels et al., 2011 [67] | GHB | DBS (50 µl [44] /6-mm punch [67]) | **Derivatization**:100 [44] or 50 µl [67] TFAA/HFB-OH (2:1) | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (12.3 min) | GHB-d6 | 2-100 µg/ml |  |
| Ingels et al., 2013[41] | GHB | UrinePlasma/serumBlood/lyzed blood(100 µl) | **PP**/salting-out: 100 mg Na2SO4**Derivatization**: 30 µl DMS + 30 µl NaOH (0.5 M)**Headspace-trap extraction of derivatized GHB**: 30 min 90 °C | 94 % dimethyl – 6 % cyanopropylphenyl polysiloxane 30 m x 0.25 mm, 1.4 µm(15 min) | GHB-d6 | 5-150 urine2-150 plasma/serum3.5-200 blood/lyzed blood |  |
| Jones et al., 2007[116] | GHB\* | Blood(0.5 g) | **PP**: acetone**Conversion GHB to GBL**: sulphuric acid **LLE** **of GBL**: dichloromethaneevaporation to 50-100 µl | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (NR) | GVL | 8-1000 µg/ml | 2 µl injectedGC-FID |
| Kankaanpää et al., 2007[105] | GHBGBL1,4-BD | UrineBlood(200 µl) | **LLE**: 400 µl water + 5 ml t-butylmethylether + 0.5g NaCl**Derivatization**:100 µl acetonitrile + 30 µl MSTFA  | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.32 mm, 1.0 µm(12 min) | benzylalcohol | 3-75 µg/ml  | *GBL determination:*similar sample prep: without salting –out and derivatization +different GC-MS method3 µl injected |
| Kerrigan, (2002) [59];Mazzar-Proo and Kerrigan, (2005)[126] | GHB | UrineBloodVitreous humour(0.5 ml) | **LLE**:250 µl 0.1 M HCl + 2 ml ethylacetate (2 x)**Derivatization**: 100 µl (BSTFA + 1% TMCS) [42]; 50 µl MSTFA [104] | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm(13 min) | GHB-d6 | 0.4-250 µg/ml | GC-PCI-MS(methane as reagent gas) |
| Kimura et al., 2003[91] | GHB | Urine(0.01mg creatinine) | Urease 37 °C 30 minAlkalinization: 10 µl 0.1 N NaOH**Derivatization**: 60 µl (BSTFA + 1 % TMCS) 80 °C 30 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (20.9 min) | dimethylsuccinic acid | 0.006-0.0127 µg/ml |  |
| Lebeau et al., 2000[8] | GHB\* | UrineBlood (1ml) | **Conversion GHB to GBL**: 0.15 ml concentrated sulphuric acid**LLE** **of GBL**: 5 ml dichloromethane **Static headspace injection**:20 min 90 °C | 94 % dimethyl – 6 % cyanopropylphenyl polysiloxane 30 m x 0.25 mm, 1.4 µm(15 min) | α-methylene- γ- butyrolactone(screening)GHB-d6 (single-point quantification) | 5-1000 µg/ml | 1) headspace GC-FID screening2) headspace GC-MS confirmationnitrogen as carrier gas |
| Lenz et al., 2009[120] | GHB\* | UrineSerum(0.5 ml) | **Conversion GHB to GBL**: 100 µl sulphuric acid**SPDE of GBL**: + 1 g sodium sulphate; SPDE PDMS/AC coating; 150 extraction strokes at 50 °C; sample agitation 500 rpm | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 1.0 µm (10.5 min)  | GHB-d6 | 2-200 µg/ml | SPDE GC-PCI-MS(methane as reagent gas) |
| Lora-Tamayo et al., 2003[123] | GHB1,4-BD | UrineBlood(1 ml) | **LLE**: 250 µl cold 0.1N sulphuric acid + 6 ml ethylacetate **Derivatization**: 30 µl (BSTFA + 1% TMCS) + 60 µl acetonitrile 70°C 15 min; + 200 µl ethylacetate | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.33 µm (14 min) | GHB-d6 | 2.5-85 µg/ml urine  |  |
| Louagie et al., 1997[112] | GHB | Serum(20 µl) | **PP**: 40 µl acetonitrile**Derivatization**: 20 µl MSTFA 90°C 10 min | 100 % polydimethylsiloxane12 m x 0.2 mm, 0.33 µm (9.6) | valproic acid | 2-200 µg/ml |  |
| Marinetti et al., 2005[31] | GHBGHV | UrineBloodVitreous humour(200 µl) | **PP**: 1 ml acetone**SPE** *(CLEAN SCREEN GHB)*: conditioning: 3 ml methanol, 3 ml deionized water, 3 ml 100 mM sodium phosphate buffer (pH 6); sample loading (reconstituted in 250 µl 100 mM sodium phosphate buffer (pH 6)); elution: 1 ml 99:1 methanol/ammonium hydroxide**Derivatization**: 100 µl (BSTFA + 1 % TMCS) 55 °C 30 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (13.0 min) | GHB-d6 | 5-150 µg/ml | Post-mortem cases |
| McCusker et al., 1999[43] | GHB | Urine (200 µl) | **SPE** *(CLEAN SCREEN GHB)*:conditioning: 3 ml methanol, 3 ml deionized water, 0.5 ml sodium phosphate buffer (pH 6); sample loading ; elution: 1 ml 99:1 methanol/ammonium hydroxide**LLE**: DMF + hexane**Derivatization**: 100 µl ethylacetate + 100 µl (BSTFA+ 1% TMCS) 60 °C 5 min | 100 % polydimethylsiloxane30 m x 0.25 mm, 0.25 µm (10.97 min) | GHB-d6 | 5-500 µg/ml |  |
| Meyer et al., 2010[35] | GHB, DL-lactic acid, glycolic acid, ethylene glycol, and other glycols | UrinePlasma(50 µl) | **PP**: 50 µl acetonitrile**Derivatization**: 300 µl BSTFA + 20 µl DMF | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (12 min) | GHB-d6 | 10-200 µg/ml | Micro-wave assisted silylation |
| Pan et al., 2001[37] | GHBEthyleneglycol | Urine Blood(100 µl) | **PP**: 80 µl water + 200 µl acetonitrile**LLE**: supernatant 150 µl + 500 µl DMP/DMF (80:20) **Derivatization**: 50 µl MTBSTFA + 50 µl ethylacetate 70°C 60 min | 5 % phenyl – 95 % methylpolysiloxane25 m x 0.25 mm, 0.25 µm (16.3 min) | 2-hydroxy-3-methylbutyric acid | 0-500 µg/ml | 6 case histories |
| Paul et al., 2006[33] | GHB | UrineBlood(50 µl) | **PP**: 150 µl acetonitrile +50 µl 0.1 M sulphuric acid **Derivatization**: 100 µl BSTFA 60 °C 20 min | 5 % phenyl – 95 % methylpolysiloxane15 m x 0.25 mm, 0.25 µm (9.40 min) | GHB-d6 | 2.5-100 µg/ml  | GC-EI-MS/MS  |
| Sakurada et al., 2002[124] | GHB1,4-BDSSABHB | UrineBlood(1 ml) | **LLE**: 400 µl cold 0.1N sulphuric acid + 8 ml ethylacetate **Derivatization**: 50 µl (BSTFA + 1% TMCS) + 60 µl acetonitrile 70°C 15 min;+ 40 µl ethylacetate | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (14 min) | diethyleneglycol | 0-200 µg/ml | 2 µl injected |
| Shima et al., 2005[32] | GHBBHBAHB | Urine(100 µl) | **PP**: 500 µl methanol**Derivatization**: 50 µl (BSTFA + 1% TMCS) + 50 µl acetonitrile 60 °C 20 min | 50 % phenyl - methylpolysiloxane30 m x 0.32 mm, 0.25 µm(17 min) | 2-hydroxycaproic acid | 0.01-30 µg/ml |  |
| Van hee et al., 2004[36] | GHB, DL-lactic acid, glycolic acid, ethylene glycol, and other glycols | UrinePlasmaSerum(20 µl) | **Derivatization**: 750 µl BSTFA + 20 µl DMF 70°C 15 min  | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm (9.25 min) | GHB-d6 | 3.2-200 µg/ml | Split injection (60:1) |
| Villain et al., 2003[16] | GHB | UrineBlood(20 µl) | **PP**: 45 µl acetonitrile**Derivatization**: 35 µl BSTFA + 1% TMCS 70 °C 25 min | 5 % phenyl – 95 % methylpolysiloxane30 m x 0.25 mm, 0.25 µm(11.5 min) | GHB-d6 | 1-200 µg/ml |  |

LC

| Ref. | Analyte | Matrix (sample volume) | Sample preparation | Stationary phase(total run time)  | IS GHB | Calibration Range GHB | Remarks  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Dahl et al., 2012[99] | GHBGBL1,4-BDPregabalineBHB | UrineBlood(100 µl) | Urine: **Dilution**/**Filtration**:1) 1:1 dilution: + 100 µl water2) 1.75 ml 0.2 % formic acid3) 500 µl: mini-UniPrep filter 0.2 µm filter membraneBlood:**Dilution**: + 100 µl water**PP**: 400 µl ice-cold acetonitrile: methanol (85:15, v/v), 10 min freezer**SPE**: 96-well plate (30 mg OASIS HLB): conditioning: methanol and water; sample loading: supernatant + 250 µl 0.4 % formic acid solution  | HSS T3 column (100 mm x 2.1 mm, 1.7 µm) gradient elutionMP A: 0.2 % formic acid MP B: methanol (4.5 min) | GHB-d6 | 2.6-312.3 µg/ml blood | UHPLC-MS/MSESI(+)Injection volume:3 µl blood/2 µl urine |
| de Vriendt et al., 2001[108] | GHB | Rat plasma (60 µl) | **SPE** (SAX Bond elut cartridges):conditioning: 1 ml methanol, 6 ml 0.5 M formic acid, 1 ml water; sample loading: 60 µl; washing: 0.5 ml water, 0.5 ml water-methanol (1:1), 0.3 ml methanol; elution: 600 µl acetonitrile with 6 % acetic acid |  C18 Aqua column (150 mm x 4.6mm, 5µm)isocratic elution MP: 100% potassium dihydrogenphosphate solution 20mM(10 min) | (tested different IS) | 10-750 µg/ml  | HPLC-UV (220 nm)injection volume: 100 µl |
| Dresen et al., 2007[113] | GHB | Serum(100 µl) | **PP**: 200 µl cold acetonitrile | Polar-enca**pp**ed phenylpropyl RP (Synergy Polar-RP)(50 mm x 2 mm, 4 µm)gradient elution MP A: 0.1 % formic acid with 1 mmol/L ammonium formateMP B: acetonitrile with 0.1 % formic acid 95:5 (v/v) with 1 mmol/L ammonium formate (15 min) | GHB-d6 | 1-200 µg/ml | HPLC-MS/MSESI(-)injection volume: 20 µlNo evaluation of matrix effect |
| Dziadosz et al., 2013[117] | GHB | Serum(100 µl) | **PP**: 1 ml water/methanol (3/97 v/v) 15 min shaking | Luna 5 µm C18 (2) 100 A (150 mm x 2 mm)gradient elution MP A: water/methanol (95/5)+ 10 mM ammonium acetate + 0.1 % acetic acid (pH 3.2)MP B: water/methanol (3/97)+ 10 mM ammonium acetate + 0.1 % acetic acid (pH 3.2)(3 min) | GHB-d6 | 5-100 µg/ml | UHPLC-MS/MS ESI(-)injection volume: 10 µlAdduct-supported detection |
| Elian and Hackett, 2011[107] | GHB | Urine (50 µl) | **Dilution**: 1ml water, mix, + 3 ml water**SPE** (SAX, CUQAX 6 ml 500 mg):conditioning: 3 ml methanol, 3 ml water; sample loading; washing: 3 ml deionized water, 3 ml methanol; elution: 3 ml 6 % acetic acid in methanol | Allure biphenyl (150 mm x 4.6 mm, 5 µm)gradient elutionMP A: 0.1 formic acid in waterMP B: 0.1 % formic acid acetonitrile(4.1 min) | GHB-d6 | 0.5-10 µg/ml | HPLC-MS/MSAPCI(-)injection volume: 10 µl |
| Forni et al., 2013[68] | GHB | DBS(3 x 4.6 mm) | **Extraction**: 200 µl methanol 30 min | HSS T3 column (100 mm x 2.1 mm, 1.8 µm) gradient elutionMP A: 0.1 % formic acid in waterMP B: 0.1 % formic acid in acetonitrile(4 min) | GHB-d6 | 1-128 µg/ml | UHPLC-MS/MSESI(-)injection volume: 15 µl |
| Fung et al., 2004[129] | GHB | Rat plasma(100 µl) | **SPE** (SAX Bond elut cartridges, 100 mg 1 ml): conditioning: 1 ml methanol, 6 ml 10 % acetic acid, 1 ml water; sample loading: 100 µl; washing: 0.5 ml water, 0.5 ml water-methanol (1:1), 0.3 ml methanol; elution: 3 ml acetonitrile with 10 % acetic acid | C18 Aqua column (150 mm x 4.6mm, 5µm)gradient elutionMP A: 90 % 5mM formic acid MP B: 10 % acetonitrile(5 min) | GHB-d6 | 0.1-10 µg/ml | HPLC-MS/MS APCI(-)injection volume: 10 µl |
| Johansen and Windberg, 2011[102] | GHBGBL1,4-BDGVL | Urine(20 µl)Blood(0.2 g) | Blood:**PP**: 260 µl acidic methanol **Dilution**: 50 µl supernatant diluted 1:1 acidic water Urine:**Dilution**: 10-fold dilution with acidic 10 % methanol | Zorbax SB C18 (150 mm x 2.1 mm, 3.5 µm) gradient elutionMP A:acidic water MP B: acidic methanol(19 min) | GHB-d6 | 1-100 mg/kg blood | HPLC-MS/MSESI(+)injection volume : 5 µl |
| Kaufmann and Alt, 2007[128] | GHB | UrineSerum(250 µl) | **LLE**:1) 125 µl 0.1 M HCl + 1 ml ethylacetate 2) 750 µl t-butylmethylether**Derivatization**: 50 µl 3 M HCl n-butanol 50 °C 5 min | C18 zorbax SB-18 Agilent (30 mm x 2.1 mm, 3.5 µm)gradient elutionMP A: 5 mM ammonium formate in waterMP B: 5 mM ammonium formate in acetonitrile(NR) | GHB-d6 | 2-100 µg/ml | HPLC-MS/MSIon trap injection volume: 10 µl |
| Lott et al., 2012 [114] | GHB | Serum(100 µl) | **PP**: 200 µl acetonitrile | Nucleodur HILIC column (NS, 3 µm)isocratic elution80 % acetonitrile 20 % water with 5 mM ammonium acetate(15 min) | GHB-d6 | 2-50 µg/ml | HILIC, HPLC-MS/MS APCI(-)injection volume: 10 µl |
| Sørensen and Hasselstrøm, 2012[109] | GHBGBL1,4-BD | Blood (200 µl) | **PP**: + 100 µl methanol + 600 µl acetonitrile **SPE** (3- ml strata-X-C 60 mg SCX): conditioning: 1 ml methanol, 1 ml water, 1 ml 1 M sodium dihydrogen phosphate solution, 1 ml water; sample loading: 600 µl supernatant + 250 µl water  | SeQuant ZIC HILIC (100 mm x 2.1 mm, 5 µm) gradient elutionMP A: 1mM ammonium acetateMP B: acetonitrile(10 min) | GHB-d6 | 5-200 µg/ml | HILIC, UHPLC-MS/MS ESI(+) and ESI(-)injection volume: 10 µl |
| Wood et al., 2004[34] | GHBGBL1,4-BD | Urine(250 µl) | **Dilution**: 1:20 with deionized water  | Atlantis C18 column (100 mm x 3 mm, 5 µm)isocratic elutionMP: 0.1 % aqueous formic acid: methanol (90:10)(11 min) | GHB-d6 | 1-80 µg/ml | HPLC-MS/MSESI(+)injection volume: 20 µl |
| Zacharis et al., 2008[88] | GHB | Oral fluid(500 µl) | Saliva sample evaporated; residu reconstituted 200 µl DMF (water removal using preheated molecular sieves)**Derivatization**: 1) 100 µl Br-MMC 70 °C 70 min2) 100 µl anthracene + 1ml acetonitrile | C18 Kromasil(250 mm x 4.6 mm, 4µm)gradient elutionmethanol/phosphate buffer (40 mM, pH 3)(25 min) | anthracene | 0.25-7.5 µg/ml  | HPLC-Fluorescence detection (330 (λex) -390 (λem) nm)injection volume: 25 µl |

Other confirmation method

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Ref. | Analyte | Matrix (sample volume) | Extraction | Separation buffer  | Calibration Range GHB | Remarks  |
| Gong et al., 2008[103] | GHBBHBAHB | UrineSerum  | **Dilution**: 1:4 with separation buffer | 20 mM arginine; 10 mM maleic acid; 30 µM cetyltrimethylammoniumbromide5 mM vancomycin | 2-400 µg/ml urine5-150 µg/ml serum | Capillary electrophoresis with contactless conductivity detection |

1,4-BD: 1,4-butanediol; AHB: alpha-hydroxybutyric acid; APCI: atmospheric pressure chemical ionization; BHB: bèta-hydroxybutyric acid; Br-MMC: 4-bromomethyl-7-methoxy coumarin; BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide; CW/TPR: carbowax/templated resin; DMF: dimethylformamide; DMS: dimethylsulphate; EI: electron impact; ESI: electrospray ionization; FID: flame ionization detection; GBL: gamma-butyrolactone; GC: gas chromatography; GHB: gamma-hydroxybutyric acid; GHV: gamma-hydroxyvaleric acid; GVL: gamma-valerolactone; HCl: hydrogen chloride; HFB-OH: heptafluorobutanol; HILIC: hydrophilic interaction liquid chromatography; HPLC: high performance liquid chromatography; IS: internal standard; KET: ketamine; LLE: liquid-liquid extraction; MAMP: methamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; MP: mobile phase; MS: mass spectrometry; MS/MS: tandem mass spectrometry; MSTFA: N-methyl-N-(trimethylsilyl)-trifluoroacetamide; MTBSTFA: N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide; NaCl: sodium chloride; NaOH: sodium hydroxide; Na2SO4: anhydrous sodium sulphate; NCI: negative chemical ionization; NR: not reported; PCI: positive chemical ionization; PDMS: polydimethylsiloxane; PDMS/AC: PDMS/cyanopropyl; PP: protein precipitation; RP: reversed phase; SPDE: solid-phase dynamic extraction; SPE: solid-phase extraction; SPME: solid-phase micro-extraction; SSA: succinic semi-aldehyde; TBCS: butyldimethylchlorosilane; TFAA: trifluoroacetic acid anhydride; TMCS: trimethylchlorosilane; UHPLC: ultra high performance liquid chromatography; UV: ultraviolet

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