Anal Bioanal Chem (2014) 406:3553–3577
DOI 10.1007/s00216-013-7586-6

REVIEW

Screening and confirmation methods for GHB determination in biological fluids

Ann-Sofie M. E. Ingels · Sarah M. R. Wille · Nele Samyn · Willy E. Lambert · Christophe P. Stove

Received: 31 October 2013 / Revised: 9 December 2013 / Accepted: 14 December 2013 / Published online: 6 February 2014

Abstract The purpose of this review is to provide a comprehensive overview of reported methods for screening and confirmation of the low-molecular-weight compound and drug of abuse gamma-hydroxybutyric acid (GHB) in biological fluids. The polarity of the compound, its endogenous presence, its rapid metabolism after ingestion, and its instability during storage (de novo formation and interconversion between GHB and its lactone form gamma-butyrolactone) are challenges for the analyst and for 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 liquid chromatography and capillary zone electrophoresis have also been used. Before injection, sample-preparation techniques include (a combination of) liquid–liquid, solid-phase, or headspace extraction, and chemical modification of the polar compound. Also simple "dilute-and-shoot" may be sufficient for urine or serum. 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

Published in the topical collection *Forensic Toxicology* with guest editor Helena Teixeira.

A.-S. M. E. Ingels · W. E. Lambert · C. P. Stove (✉)
Laboratory of Toxicology, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium
e-mail: Christophe.Stove@UGent.be

A.-S. M. E. Ingels · S. M. R. Wille · N. Samyn
Laboratory of Toxicology, National Institute of Criminalistics and Criminology, Federal Public Services, Vilvoordsesteenweg 100, 1120 Brussels, Belgium

Introduction

Gamma-hydroxybutyric acid (GHB) or 4-hydroxybutanoic acid, a short chain fatty acid (pK_a 4.6–4.8) with two polar substituent groups (hydroxyl and carboxyl), 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 function as an endogenous compound remains unclear; it probably acts as a neuromodulator or neurotransmitter via $GABA_B$ 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 anesthetic, but this use has been abandoned in most countries because of side effects such as seizures and amnesia [6]. Furthermore, GHB and/or GBL-containing supplements were sold to increase body muscles—because it is assumed 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 for treatment of narcolepsy with cataplexy and excessive daytime sleepiness (Xyrem) and of alcohol (Alcover, Italy) and 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 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 in bottles mixed with, e.g., water, from which typically a capful is ingested orally per dose [4]. GHB abuse has also been

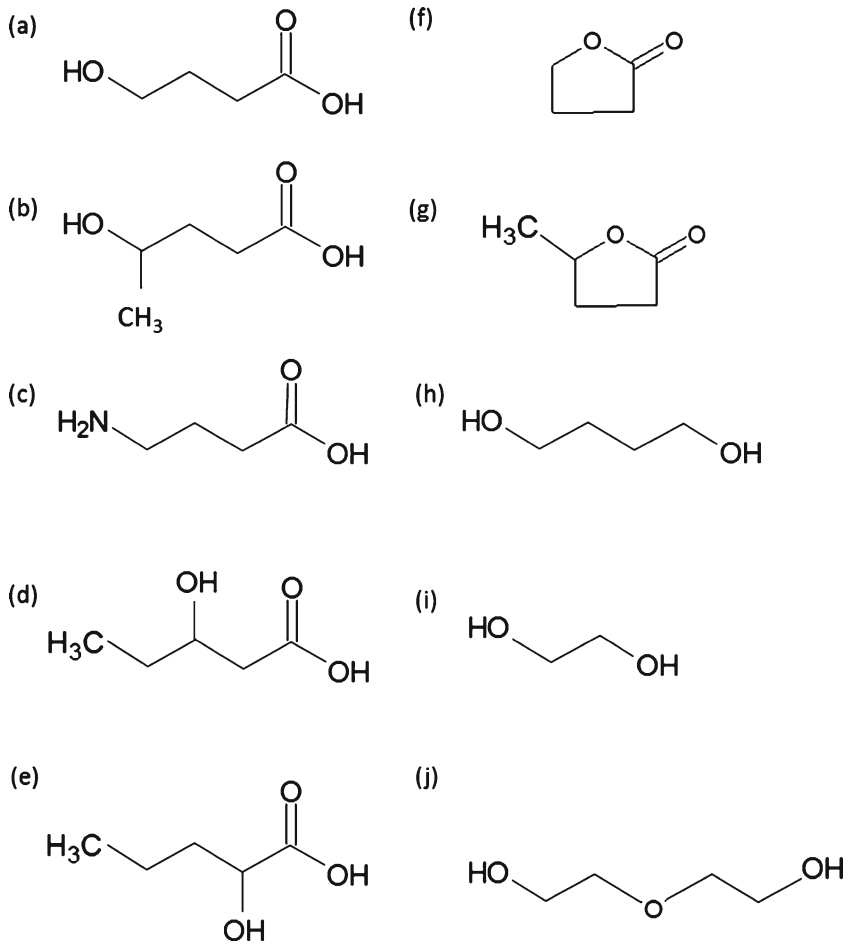


Fig. 1 Structures of gamma-hydroxybutyric acid (GHB; *a*), gamma-hydroxyvaleric acid (GHV; *b*), gamma-aminobutyric acid (GABA; *c*), beta-hydroxybutyric acid (BHB; *d*), alpha-hydroxybutyric acid (AHB; *e*), gamma-butyrolactone (GBL; *f*), gamma-valerolactone (GVL; *g*),1,4-butanediol (1,4-BD; *h*), ethylene glycol (EG; *i*), and diethylene glycol (DEG; *j*)

reported in drug-facilitated sexual assaults (DFSA), because of its strong sedative and amnesic effects. The possibility of rendering a victim unconscious is enabled by the chemical properties of GHB—a colorless 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^{-1} body weight. [5]. Euphoria, relaxation, increased sociability, and reduced psychomotor skills are among the effects experienced when using GHB; these effects are similar to those 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]. Also, the effects reported after 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 such as relaxation and euphoria whereas doses of 2.5 to 3 g may lead to side-effects such as nausea and vomiting. Higher doses (3 to 4 g) may result in loss of consciousness and a dose of more than 4 g can result 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 as a result of the use of GHB alone or in combination with other drugs, for example alcohol and ecstasy [11].

First reports of abuse appeared in the early 1990s [12, 14]. Since the late 1990s and early 2000s the incidence of GHB intoxication has apparently been decreasing worldwide. For example, when GHB exposure reported to the California Poison Control System from 1999 to 2003 was evaluated, a decrease was recorded; this may reflect the true incidence, but may also be because of a decrease in adverse events without a decrease in overall GHB use; it may also be a result of random variability [14]. According to 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—remain 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 was not routinely available until 2009. In addition, because of the rapid elimination of GHB from the human body, delayed sampling may result in GHB concentrations below current cut-off values, thus no longer enabling 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 intoxication at raves or other dance parties [11, 17].

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

Since the late 1990s, the Food and Drug Administration (FDA) has banned all non-prescription sales of GHB. Illicit GHB is, since 2000, a Schedule I agent (Controlled Substances Act) in the US. In 2002 the FDA approved the use of sodium oxybate for 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 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, manufacture, or sale with the intention of 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].

A variety of bioanalytical methods for GHB determination has 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 by use of a second, independent method, mostly involving quantification [28]. This has been achieved mostly by gas chromatography (GC), although liquid chromatography (LC) and capillary zone electrophoresis (CZE) have also been used.

Analytes of interest

Although in a toxicological context it might be relevant to determine whether GHB positivity is the result of intake of GHB, GBL, or 1,4-BD, GHB remains the most important analyte to search for in biological fluids, owing to the rapid in-vivo biotransformation of its precursors [29, 30]. Also after 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 for simultaneous determination (i.e. 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 ethylene glycol (EG) is suspected, the latter also causing high anion gap metabolic acidosis; Fig. 1) [35–37], and other club drugs, for example MDMA, ketamine [38], and gamma-hydroxyvaleric acid (GHV) or its lactone, GVL (reported to be a GHB alternative) [31, 39] (Fig. 1).

It must be kept in mind that a quantitative result may be affected 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 and/or 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 [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 and GBL are completely converted to GBL and GHB, respectively, before analysis [8, 41, 42]. Second, conversion did not occur, so absolute GHB was measured [16, 35, 36]; last, conversion occurred but was minimal, with little or no relevance in the forensic or clinical setting [43, 44]. Therefore, the method of analysis must be considered when comparing 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 because of conversion to GBL of some of the (endogenous) GHB present in post-mortem plasma or urine during storage, depending on sample pH [45, 46].

GHB concentrations and matrices of interest

As mentioned above, the natural presence of GHB results in measurable baseline levels in a variety of biological matrices. Studies have been conducted on, e.g., urine [47–50], plasma [49], serum [50] whole blood [32, 48, 49], and oral fluid [51] samples obtained from healthy non-users. Data from non-GHB related fatalities [45, 52] and concentrations arising from exogenous administration have also been collected. Ingestion can be intentional—for recreational or therapeutic use—or accidental; both may lead to overdoses or even fatalities, as illustrated by several case reports [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, for whom there is a narrow range between recreational doses and overdoses. An overlap between highly toxic and lethal concentrations has been observed, with high inter-individual variability of the relationship 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^{-1} GHB in plasma may be sufficient to cause death [58]. In addition, elevation of GHB concentrations during storage of urine and blood has been reported, further complicating interpretation of a GHB concentration. Appropriate storage of samples until analysis is therefore required (recommendation: −20 °C) [9, 13, 46, 57, 59]. De-novo formation in post-mortem blood has also been reported. For more detailed information about GHB production in post-mortem cases we refer the interested reader to the literature [45, 52, 60, 61].

To differentiate between endogenous and exogenous concentrations [49], cut-off levels have been established. Most authors agree on a 10 μg mL^{-1} cut-off level for GHB in ante-mortem urine [48, 49, 59], although suggestions of 5 [62] or 6 [50] μg mL^{-1} have also been made. For ante-mortem whole blood, 10 [16, 49], 5 [48] or 4 [50] μg mL^{-1} has been proposed as a cut-off, and one group even proposes 1 μg mL^{-1}, if appropriate storage is guaranteed [32]. This implies that screening and confirmation methods for GHB in ante-mortem urine, whole blood, and plasma should, ideally, have a decision limit or lower limit of quantification (LLOQ) below or equal to 4 or 5 μg mL^{-1}. Higher cut-off levels have been proposed for post-mortem matrices (20 for urine, 30 and 50 for whole blood, and 12 μg mL^{-1} for vitreous humor) 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 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^{-1}) prepared in authentic matrices [33], and complicates interpretation of a positive result.

As an alternative to the use of interpretative cut-off concentrations, continuous-flow GC–combustion–isotope-ratio MS has been used to discriminate between exogenous (i.e. synthetic) and endogenous GHB in blood. Initial findings suggest different ^{13}C and ^{12}C content of the endogenous and synthetic form of GHB [64]. However, it is obvious that the cost and complexity associated with this complex technique severely limits its general applicability.

In addition to the endogenous presence and possible instability of GHB during storage, samples must be collected as soon as possible after ingestion, because of the extensive metabolism of GHB once ingested orally (plasma $T_{1/2}$ less than 1 h) [5, 65, 66]. Otherwise, GHB concentrations in blood and urine will drop to endogenous levels within 6 to 12 h after intake, no longer enabling proof of intake of GHB, possibly leading to 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, and unconventional matrices such as sweat and oral fluid. Only moderate results have been obtained by use of the latter two matrices because diffusion of the acidic drug in these has been shown to be limited. After GHB intake (50 mg kg^{-1} 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 and plasma inter-variability. GHB concentrations in sweat were only slightly higher than baseline values [44, 66–70]. Hair analysis has, in contrast, been shown useful for extending the window of detection, because of incorporation of GHB in the hair matrix. A case report has described detection of DFSA even after a single use only [71]. Also in hair, endogenous GHB is present, often rendering it difficult to draw straightforward conclusions. Therefore, small segments are analyzed to detect elevation of baseline GHB concentration as a result of exogenous ingestion [72, 73].

More than 95 % of an oral dose of GHB is converted to CO_2 and H_2O 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. [74] demonstrated the existence of a new metabolite, GHB-glucuronide, in urine, in concentrations ranging from 0.11 to 5.0 μg mL^{-1}. Although more research, for example pharmacokinetic studies after 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].

Procedures for screening for the presence of GHB in biological fluids

A good screening procedure enables simple, sensitive, selective, and rapid identification of unknown analytes in a minimal amount of sample. STA approaches typically use immuno- and/or enzymatic assays to screen for analytes or categories of compounds, then GC–mass spectrometry (GC–MS) or high-performance liquid chromatography–diode array detection (HPLC–DAD) for high-throughput 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 increasing interest nowadays, sometimes even replacing the immunological and/or enzymatic tests [28, 75, 76]. An overview of possible screening procedures for GHB is given below, 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 also be used for screening. A screening method preferably has a decision limit (cut-off of the applied assay) at or below the exogenous and/or endogenous cut-off, to enable reliable initial differentiation between samples considered to be GHB-positive or negative. However, because GHB concentrations in moderately to severely intoxicated GHB patients, for example those brought to an emergency department in a comatose state, will usually be well above these cut-off levels, we also consider in this review methods with decision limits and/or LLOQs (well) above these cut-offs as screening methods. As with any screening test, a positive result should only be regarded as a preliminary indication and must be confirmed by use of an independent, preferably MS-based, technique, for example GC–MS or LC–MS(–MS).

Colorimetric tests

Badcock and Zotti [78] reported a colorimetric test that enables identification of GHB in human urine on the basis of the conversion of GHB to GBL. Briefly, after addition of concentrated sulfuric acid, ammonium sulfate, and nitroprusside to 250 μL urine, an intense and instant blue–olive-green color 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 coloring 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^{-1} [78, 79].

Enzymatic assays

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

Colorimetric enzymatic assays

Bravo et al. [80] developed a solution-endpoint and a dipstick-assay for determination of GHB in human urine. Identification was possible by coupling the oxidation reaction of GHB, via 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^{-1} GHB is present in urine.

Another test strip, commercially available from Drugcheck, can detect GHB in human urine with a cut-off level of 10 μg mL^{-1}. Results are obtained within 10 min, and a color chart on the test strip is used for interpretation, next to a test strip for vitamin C, a compound with 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].

Enzymatic kit

It became clear from the tests mentioned above that more sensitive, semi-quantitative, rapid, and simple screening to detect GHB in urine and serum was urgently needed. 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 sample. Quantification is performed by using two calibrators and two quality controls provided by the manufacturer; the working range is from 5 to 250 μg mL^{-1}. Results are obtained in approximately 10 min and interferences and cross-reactivity have been evaluated. Interference by GBL of 4 % has been observed; this is

stated to have no relevant implication because GBL is rapidly converted to GHB once ingested. Also per 1.06 g L^{-1} ethanol, a 3.0 μg mL^{-1} linear increase of false-positive GHB concentration was observed, so GHB concentrations of 8–20 μg mL^{-1} need careful interpretation, especially because GHB is commonly ingested with alcoholic beverages [11]. A cut-off level of 10 μg mL^{-1} for serum and 15 μg mL^{-1} for urine has been proposed [82, 83].

Grenier et al. [84] evaluated use of this enzymatic assay as a screening method for forensic matrices including whole blood and vitreous humor. When correlating results for a variety of cases (sexual assaults, impaired drivers, and deaths) with those from a GC–MS reference method, no false negatives and few false positives were observed, with post-mortem samples seeming 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 with chromatography-based procedures. In addition, although very efficient GC–MS and LC–MS–MS procedures have been developed for GHB, integration with other tests on automated analyzers makes this assay valuable for (clinical) toxicology labs. However, Grenier et al. [84] found that a limitation of this test is that it may not be applicable to other matrices, e.g. vitreous humor, because of the high incidence of false positives.

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

Other screening techniques

^{1}H nuclear magnetic resonance (NMR) spectrometry has been used to detect GHB in urine and serum [85] and in oral fluid (600 μL) [75]. This technique is non-destructive and, because little or no sample preparation is required, is less labor-intensive than other techniques. Similarly, ion mobility spectrometry (IMS) has promise as a screening method for GHB and related compounds in urine [86]. Via direct injection using a split–splitless injector 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^{-1}.

In addition, CZE with indirect ultraviolet (UV) detection is capable of detecting high concentrations of GHB in urine after simple 1:4 dilution with water. Calibration curves ranged from 80 to 1,280 μg mL^{-1} [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 after 1:8 dilution with 3 mmol L^{-1} NaOH, completely converting GBL to GHB (calibration curve ranged from 25 to 500 μg mL^{-1}) [90]. Although accurate and precise results may be obtained by use of CZE, the LLOQ is relatively high (ranging from 25 to 80 μg mL^{-1} and 5 to 60 μg mL^{-1}, depending on urine density), compared with chromatographic techniques (LLOQ ranging from 0.1 to 8 μg mL^{-1}). Therefore, these CZE-based methods are regarded as more suitable as an alternative screening method for a GHB overdose, being rapid and simple, rather than as a secondary confirmatory method.

Chromatographic screening techniques

Compared with colorimetric and enzymatic assays, chromatographic assays typically require more intensive and time-consuming sample preparation, for example derivatization or conversion to GBL (see below). For example, Lebeau et al. [8] opted for a gas chromatography–flame ionization detection (GC–FID) screening method using headspace (HS) as injection technique after conversion of GHB to GBL; confirmation of GHB (as GBL) was by GC–MS. Also, in clinical practice, in which the objective is to achieve medical diagnosis and start treatment, non-specific detection such as by GC–FID is sufficient, as stated by Blanchet et al. [42]. These authors determined GHB after derivatization with BF_3–butanol.

Similarly, urinary organic acid assays based on silylation and GC–MS, more readily available than GHB assays in hospital laboratories, were investigated for detection of GHB in urine. However, if these methods included acidification of the samples during sample treatment, which favors conversion of GHB to GBL, only a small GHB peak was visible, as might be expected [91]. In addition, silylated urea may elute close 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 identification of GHB with higher confidence [92–94].

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

An LC–MS–MS method for screening of DBS from newborns for elevated GHB concentrations has recently been reported. This enables diagnosis of SSADH deficiency, a rare inherited metabolic disorder in which GHB concentrations are increased because of a deficiency of the succinic semialdehyde dehydrogenase enzyme responsible for conversion of SSA to succinate [68]. Although not intended for

toxicology purposes, this method may also be applicable to screening of DBS for exogenous GHB [97, 98].

In addition to these screening methods, several authors have reported simplified and rapid procedures for high-throughput determination of GHB, leading to the possibility of using confirmation methods for screening as well. 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. [36] determined GHB (and glycols) in small volumes of plasma and urine (20 μL) by use of GC–MS, by adding excess silylation reagent directly to the biological sample. This procedure was recently modified by Meyer et al. [35], who used microwave-assisted derivatization; this is another approach particularly useful in the laboratories of hospital emergency departments, because quantitative results for urine can be obtained within 30 min by use of one-point calibration. Other examples of procedures with minimum hands-on time are those in which derivatization reagents are applied directly "on spot" (for DBS) or "in-vial" (in 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 for screening because of easy sample preparation and resulting high-throughput [99].

Confirmation methods for clinical and forensic cases

Methods suitable for confirmation of an assumed GHB-positive sample ideally have 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. Because 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 usually more complicated than that used for colorimetric or enzymatic methods, which are primarily suitable for urine and serum. Below, an overview of commonly used sample-preparation procedures is given, followed by an overview of the analytical techniques used to separate and detect GHB (and analogues). Table 1 provides an overview of the different published procedures. To evaluate if a given method enables differentiation between exogenous and endogenous GHB, the calibration range with the quantification limit is included. Also the choice of internal standard may affect data quality and has, therefore, also been included in the table [100]. As shown in the table, several compounds with similarity to GHB have been used as internal standards. In MS-based methods, use of a deuterated internal standard is recommended to compensate for variations during sample preparation and analysis. The deuterated form of GHB, GHB-d6, has been used widely for this purpose; a C-labeled internal standard is not yet commercially available.

Sample preparation

The techniques used to treat biofluids comprise dilution, filtration, deproteinization, chemical modification, liquid–liquid extraction (LLE), solid-phase extraction (SPE), and HS extraction, sometimes alone but usually combined. 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, for example dilution and direct derivatization ("on spot" and "in-vial"), together with microwave-assisted derivatization and on-line derivatization techniques, for example injection port derivatization. Some of these simplifications have been made possible by the introduction of improved separation and detection techniques, for example tandem MS, resulting in procedures with minimal hands-on time. In addition, the initial sample volume required for analysis may be reduced without loss of method sensitivity. The latter also depends on GHB recovery, a method property worth evaluating during optimization of sample treatment. Recovery should be reproducible and sufficient in terms of method sensitivity. Therefore, examples of strategies affecting recovery are also mentioned below.

Dilution and filtration of the biological fluid

If appropriate separation and detection techniques are used, simple dilution of urine and serum, with or without subsequent filtration, may be sufficient as sample preparation [34, 99, 102]. This has been demonstrated for 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] before LC–MS–MS analysis. As an alternative, urine and serum have been diluted 1:4 with a buffer solution before CZE analysis with contactless conductivity detection (CZE–C^4D) [103].

It is important to note that sensitivity must be sufficient, as illustrated by Wood et al. [34], who compared method sensitivity (measured as signal-to-noise ratio, *S/N*) for two sample-pretreatment procedures before LC–MS–MS analysis. The first of these procedures was 1:20 dilution of urine (with deionized water containing internal standard); the second was based on a more time-demanding SPE extraction (OASIS

Table 1 Overview of confirmation methods for determination of 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, ionization mode EI, and helium as carrier gas. *If method includes conversion of GHB to GBL without acidification of the sample, determination of original GBL concentration is possible)

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase m×mm, μm (total run time)	IS GHB	Calibration range GHB μg mL^{-1}	Remarks
Abanades et al., 2006 [5]; Abanades et al., 2007 [66]	GHB	Urine Plasma Oral fluid (100 μL) (Sweat)	PP: 150 μL acetonitrile+50 μL 0.1 mol L^{-1} sulfuric acid Derivatization: 50 μL (BSTFA+1 % TMCS) 70 °C 30 min	5 % phenyl–95 % methylpolysiloxane 12×0.2, 0.33 (14 min)	GHB-d6	0.2–300	
Andresen et al., 2010 [50]	GHB	Urine Serum (100 μL)	LLE: 200 μL 0.1 mol L^{-1} HCl+1 mL ethyl acetate Derivatization: 100 μL ethyl acetate+50 μL (BSTFA+1 % TMCS)	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (22.7 min)	GHB-d6	2–12	
Andresen-Streichert et al., 2013 [39]	GHB GHV	Urine (100 μL)	LLE: 200 μL 0.1 mol L^{-1} HCl+1 mL ethyl acetate Derivatization: 50 μL acetonitrile+25 μL (BSTFA+1 % TMCS)	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (16.22 min)	GHB-d6	(NR for GHB)	
Blair et al., 2000 [104]	GHB	Urine (0.5 mL)	Derivatization: 40 μL pyridine+24 μL hexyl chloroformate 40 °C 5 min Solution SPME: + 2 mL deionized water+1 mL pH 7 buffer; PDMS SPME fiber 12 min; 40 °C	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (23.33 min)	GHB-d6	5–500	SPME GC-Q-trap
Bosman and Lusthof, 2004 [54]	GHB*	Urine Blood (1 mL)	PP+Conversion of GHB to GBL: 1 mL 1 mol L^{-1} perchloric acid 1 mL supernatant: 80 °C 20 min LLE of GBL: 300 mg NaCl, pH 6.5 (1 mL 0.5 mol L^{-1} phosphate buffer+350 μL 2.5 mol L^{-1} NaOH), 5 mL chloroform	100 % polydimethylsiloxane 12×0.2, 0.33 (15 min)	GHB-d6	5–40	Case reports Split 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 % trifluoroacetic acid, 75 °C 1 h LLE of GBL: 0.55 mL 2 mol L^{-1} NaOH (pH adjusting to 6.5)+3 mL chloroform	5 % phenyl–95 % methylpolysiloxane 25×0.2, 0.33 (10.1 min)	GVL	10–50	4 μL injected
Brown et al., 2007 [38]	GHB MAMP MDMA KET	Urine (1 mL)	Derivatization: 10 μL hexyl chloroformate+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 % methylpolysiloxane 30×0.25, 0.10 (12 min)	GHB-d6	0.1–20	SPME
Chen et al., 2003 [111]	GHB	Plasma (100 μL)	PP: 2 mL acetonitrile 50 μL supernatant evaporated Derivatization: 100 μL BSTFA 75 °C 15 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 1 (9 min)	GHB-d6	2.5–250	GC–PCI–MS (ammonia as reagent gas)

Table 1 (continued)

Couper and Logan, 2000 [121]	GHB	Urine Plasma (1 mL)	LLE: 250 μL cold 0.05 mol L^{-1} sulfuric acid+6 mL ethyl acetate (2×) Derivatization: 30 μL (BSTFA+1 % TMCS)+60 μL acetonitrile 70 °C 15 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.33 (14 min)	Diethylene glycol	1–200 (urine) 1–100 (plasma)	2 μL injected
Crookes et al., 2004 [62]	GHB	Urine (2 mL)	LLE: + 0.36 mol L^{-1} sulfuric acid to obtain pH 2.75; + 3 mL ethyl acetate Derivatization: 100 μL (BSTFA+1 % TMCS) 60 °C 30 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (26.2 min)	GHB-d6	0.2–5	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 % methylpolysiloxane 30×0.25, 0.5 (16 min)	GHB-d6	0.5–50	Split injection (10:1)
Duer et al., 2001 [115]	GHB	Urine Blood Vitreous humor (25 μL)	PP: 1.225 mL GHV methanolic solution Derivatization: 100 μL MSTFA+100 μL ethyl acetate 60 °C 30 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (24.7 min)	GHV GHB-d6	Standard addition (10–500)	*GBL determination:* conversion to GHB in alkaline conditions (using 1 mL 10 mmol L^{-1} NaOH in methanol, 1 h 60°C) following GHB analysis; *1,4-BD determination*: di(dinitrobenzoyl) derivative using HPLC–UV
Elian, 2000 [124]	GHB	Urine (50 μL)	LLE: 50 μL IS+0.5 mL saturated ammonium chloride buffer+3 mL ethyl acetate Derivatization: 50 μL (BSTFA+1 % TMCS)+50 μL ethyl acetate RT 30 min	100 % polydimethylsiloxane 12×0.2, 0.33 (9.7 min)	GHB-d6	2–50	2 μL injected
Elian, 2001 [126]	GHB	Blood (50 μL)	LLE: 0.5 mL saturated ammonium chloride buffer+3 mL ethyl acetate Derivatization: 20 μL (BSTFA+1 % TMCS)+80 μL ethyl acetate 70°C 20min	100 % polydimethylsiloxane 12×0.2, 0.33 (11.0 min)	GHB-d6	1–200	2 μL injected
Elie et al., 2012 [106]	GHB	Urine (300 μL)	LLE : 300 μL urine+900 μL ethyl acetate Derivatization : 50 μL acetonitrile+50 μL (MTBSTFA+1 % TBCS)	100 % polydimethylsiloxane 30×0.25, 0.25 (15 min)	*trans*-4-Hydroxycrotonic acid	0.17–1.67	Injection port silylation: split–splitless injector with programmable pneumatic control
Elliott, 2004 [56]	GHB	Urine Plasma (1 mL)	LLE: 250 μL 0.05 mol L^{-1} sulfuric acid+6.0 mL ethyl acetate Derivatization: 75 μL (BSTFA+1% TMCS) 90°C 5 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (9 min)	GHB-d6	5–200	1) GC–FID screening method for GHB identification via GBL conversion 2) GC–MS determination via derivatization (presented in detail)
Elliott, 2004 [45]; Elliott et al., 2004 [60]	GHB	Urine Blood Vitreous humor (100 μL)	LLE: 50 μL cold 0.05 mol L^{-1} sulfuric acid+0.5 mL ethyl acetate Derivatization: 75 μL (BSTFA+1 % TMCS) 90 °C 5 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (9 min)	Diethylene glycol GHB-d6	6.25–100 (urine) 1–100 (plasma)	Comparison with GC–FID method [45]

Table 1 (continued)

Ferrara et al., 1993 [27]	GHB*	Urine Plasma (2 mL)	Conversion GHB to GBL: 2 mL cold 0.8 mol L^{-1} perchloric acid, supernatant (plasma PP) 0.2 mL 6 mol L^{-1} HCl (urine) 80 °C 20 min LLE of GBL: 300 mg NaCl, 1 mL pH 6.5 (1 mL 0.5 mol L^{-1} phosphate buffer+5 mol L^{-1} NaOH), 8 mL (plasma) or 6 mL (urine) benzene	100 % polydimethylsiloxane 12×0.2, 0.33 (15.6 min)	δ-Valerolactone	2–150 (urine) 2–200 (plasma)	
Frison et al., 2000 [29]	GHB*	Urine Plasma (0.5 mL)	Conversion GHB to GBL: 100 μL cold 1.6 mol L^{-1} perchloric acid, supernatant (plasma PP) 25 μL 6 mol L^{-1} HCl (urine) 80 °C 20 min Headspace SPME of GBL: 500 mg solid phosphate buffer 50-μm CW/TPR SPME fiber; 70 °C 10 min	Acid-modified poly(ethylene glycol) phase 25×0.2, 0.3 (9.8 min)	GBL-d6	5–150 (urine) 1–100 (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 mol L^{-1} HCl LLE of GBL: 2 mL dichloromethane (2 ×) evaporation to 100 μL	poly(ethylene glycol) 30×0.32, 0.25 (11.5 min)	GBL-d6	0.01–1	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 % methylpolysiloxane 30×0.25, 0.25 (12.3 min)	GHB-d6	2–100	
Ingels et al., 2013 [41]	GHB	Urine Plasma/serum Blood/lyzed blood (100 μL)	PP/salting-out: 100 mg Na_2SO_4 Derivatization: 30 μL DMS+30 μL NaOH (0.5 mol L^{-1}) Headspace-trap extraction of derivatized GHB: 30 min; 90 °C	94 % dimethyl–6 % cyanopropylphenyl polysiloxane 30×0.25, 1.4 (15 min)	GHB-d6	5–150 (urine) 2–150 (plasma/serum) 3.5–200 (blood/lyzed blood)	
Jones et al., 2007 [116]	GHB*	Blood (0.5 g)	PP: acetone Conversion of GHB to GBL: sulfuric acid LLE of GBL: dichloromethane evaporation to 50-100 μL	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (NR)	GVL	8–1000	2 μL injected GC–FID
Kankaanpää et al., 2007 [105]	GHB GBL 1,4-BD	Urine Blood (200 μL)	LLE: 400 μL water+5 mL*t*-butyl methyl ether+0.5 g NaCl Derivatization: 100 μL acetonitrile+30 μL MSTFA	5 % phenyl–95 % methylpolysiloxane 30×0.32, 1.0 (12 min)	Benzyl alcohol	3–75	*GBL determination:* similar sample prep: without salting –out and derivatization+different GC–MS method 3 μL injected
Kerrigan, (2002) [59]; Mazzar-Proo and Kerrigan, (2005) [125]	GHB	Urine Blood Vitreous humor (0.5 mL)	LLE: 250 μL 0.1 mol L^{-1} HCl+2 mL ethyl acetate (2 ×) Derivatization: 100 μL (BSTFA+1 % TMCS) [59]; 50 μL MSTFA [125]	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (13 min)	GHB-d6	0.4–250	GC–PCI-MS (methane as reagent gas)

Table 1 (continued)

Kimura et al., 2003 [91]	GHB	Urine (0.01 mg creatinine)	Urease 37 °C 30 min Made alkaline: 10 μL 0.1 mol L^{-1} NaOH Derivatization: 60 μL (BSTFA+1 % TMCS) 80 °C 30 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (20.9 min)	Dimethylsuccinic acid	0.006–0.0127	
Lebeau et al., 2000 [8]	GHB*	Urine Blood (1 mL)	Conversion GHB to GBL: 0.15 mL concentrated sulfuric acid LLE of GBL: 5 mL dichloromethane Static headspace injection: 20 min; 90 °C	94 % dimethyl–6 % cyanopropylphenyl polysiloxane 30×0.25, 1.4 (15 min)	α-Methylene- γ-butyrolactone (screening) GHB-d6 (single-point quantification)	5–1000	1) headspace GC–FID screening 2) headspace GC–MS confirmation nitrogen as carrier gas
Lenz et al., 2009 [120]	GHB*	Urine Serum (0.5 mL)	Conversion GHB to GBL: 100 μL sulfuric acid SPDE of GBL: + 1 g sodium sulfate; SPDE PDMS–AC coating; 150 extraction strokes at 50 °C; sample agitation 500 rpm	5 % phenyl–95 % methylpolysiloxane 30×0.25, 1.0 (10.5 min)	GHB-d6	2–200	SPDE GC–PCI-MS (methane as reagent gas)
Lora-Tamayo et al., 2003 [123]	GHB 1,4-BD	Urine Blood (1 mL)	LLE: 250 μL cold 0.05 mol L^{-1} sulfuric acid+6 mL ethyl acetate Derivatization: 30 μL (BSTFA+1 % TMCS)+60 μL acetonitrile 70 °C 15 min; + 200 μL ethyl acetate	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.33 (14 min)	GHB-d6	2.5–85 (urine)	
Louagie et al., 1997 [112]	GHB	Serum (20 μL)	PP: 40 μL acetonitrile Derivatization: 20 μL MSTFA 90 °C 10 min	100 % polydimethylsiloxane 12×0.2, 0.33 (9.6 min)	Valproic acid	2–200	
Marinetti et al., 2005 [31]	GHB GHV	Urine Blood Vitreous humor (200 μL)	PP: 1 mL acetone SPE *(CLEAN SCREEN GHB)*: conditioning: 3 mL methanol, 3 mL deionized water, 3 mL 100 mmol L^{-1} sodium phosphate buffer (pH 6); sample loading (reconstituted in 250 μL 100 mmol L^{-1} sodium phosphate buffer (pH 6)); elution: 1 mL 99:1 methanol–aqueous ammonia Derivatization: 100 μL (BSTFA+1 % TMCS) 55 °C 30 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (13.0 min)	GHB-d6	5–150	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–aqueous ammonia LLE: DMF+hexane Derivatization: 100 μL ethyl acetate+100 μL (BSTFA+1 % TMCS) 60 °C 5 min	100 % polydimethylsiloxane 30×0.25, 0.25 (10.97 min)	GHB-d6	5–500	

Table 1 (continued)

Meyer et al., 2010 [35]	GHB, DL-lactic acid, glycolic acid, ethylene glycol, and other glycols	Urine Plasma (50 μL)	PP: 50 μL acetonitrile Derivatization: 300 μL BSTFA+20 μL DMF	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (12 min)	GHB-d6	10–200	Microwave-assisted silylation
Pan et al., 2001 [37]	GHB ethylene glycol	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 ethyl acetate 70 °C 60 min	5 % phenyl–95 % methylpolysiloxane 25×0.25, 0.25 (16.3 min)	2-Hydroxy-3-methylbutyric acid	0–500	Six case histories
Paul et al., 2006 [33]	GHB	Urine Blood (50 μL)	PP: 150 μL acetonitrile+50 μL 0.1 mol L^{-1} sulfuric acid Derivatization: 100 μL BSTFA 60 °C 20 min	5 % phenyl–95 % methylpolysiloxane 15×0.25, 0.25 (9.40 min)	GHB-d6	2.5–100	GC–EI-MS–MS
Sakurada et al., 2002 [2]	GHB 1,4-BD SSA BHB	Urine Blood (1 mL)	LLE: 400 μL cold 0.05 mol L^{-1} sulfuric acid+8 mL ethyl acetate Derivatization: 50 μL (BSTFA+1 % TMCS)+60 μL acetonitrile 70 °C 15 min;+ 40 μL ethyl acetate	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (14 min)	Diethylene glycol	0–200	2 μL injected
Shima et al., 2005 [32]	GHB BHB AHB	Urine (100 μL)	PP: 500 μL methanol Derivatization: 50 μL (BSTFA+1 % TMCS)+50 μL acetonitrile 60 °C 20 min	50 % phenyl - methylpolysiloxane 30×0.32, 0.25 (17 min)	2-Hydroxy- caproic acid	0.01–30	
Van hee et al., 2004 [36]	GHB, DL-lactic acid, glycolic acid, ethylene glycol, and other glycols	Urine Plasma Serum (20 μL)	Derivatization: 750 μL BSTFA+20 μL DMF 70 °C 15 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (9.25 min)	GHB-d6	3.2–200	Split injection (60:1)
Villain et al., 2003 [16]	GHB	Urine Blood (20 μL)	PP: 45 μL acetonitrile Derivatization: 35 μL BSTFA+1 % TMCS 70 °C 25 min	5 % phenyl–95 % methylpolysiloxane 30×0.25, 0.25 (11.5 min)	GHB-d6	1–200	

Table 1 (continued)

LC Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase mm×mm, μm (total run time)	IS GHB	Calibration range GHB μg mL^{-1}	Remarks
Dahl et al., 2012 [99]	GHB GBL 1,4-BD Pregabaline BHB	Urine Blood (100 μL)	Urine: Dilution/Filtration: 1) 1:1 dilution: + 100 μL water 2) 1.75 mL 0.2 % formic acid 3) 500 μL: mini-UniPrep filter 0.2 μm filter membrane Blood: 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 (100×2.1, 1.7) gradient elution MP A: 0.2 % formic acid MP B: methanol (4.5 min)	GHB-d6	2.6–312.3 blood	UHPLC–MS–MS ESI(+) 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 mol L^{-1} 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	C_{18} Aqua column (150×4.6, 5) isocratic elution MP: 100 % potassium dihydrogenphosphate solution 20 mmol L^{-1} (10 min)	(tested different IS)	10–750	HPLC–UV (220 nm) injection volume: 100 μL
Dresen et al., 2007 [113]	GHB	Serum (100 μL)	PP: 200 μL cold acetonitrile	Polar-endcapped phenylpropyl RP (Synergy Polar-RP) (50×2, 4) gradient elution MP A: 0.1 % formic acid with 1 mmol L^{-1} ammonium formate MP B: acetonitrile with 0.1 % formic acid 95:5 (*v*/*v*) with 1 mmol L^{-1} ammonium formate (15 min)	GHB-d6	1–200	HPLC–MS–MS ESI(−) injection volume: 20 μL No 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 C_{18} (2) 100 A (150×2, 5) gradient elution MP A: water–methanol (95:5)+10 mmol L^{-1} ammonium acetate+0.1 % acetic acid (pH 3.2)	GHB-d6	5–100	UHPLC–MS–MS ESI(−) injection volume: 10 μL Adduct-supported detection

Table 1 (continued)

				MP B: water–methanol (3:97)+10 mmol L^{-1} ammonium acetate+0.1 % acetic acid (pH 3.2) (3 min)			
Elian and Hackett, 2011 [107]	GHB	Urine (50 µL)	Dilution: 1 mL 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 (150×4.6, 5) gradient elution MP A: 0.1 % formic acid in water MP B: 0.1 % formic acid acetonitrile (4.1 min)	GHB-d6	0.5–10	HPLC–MS–MS APCI(−) injection volume: 10 µL
Forni et al., 2013 [68]	GHB	DBS (3×4.6 mm)	Extraction: 200 µL methanol 30 min	HSS T3 column (100×2.1, 1.8) gradient elution MP A: 0.1 % formic acid in water MP B: 0.1 % formic acid in acetonitrile (4 min)	GHB-d6	1–128	UHPLC–MS–MS ESI(−) injection volume: 15 µL
Fung et al., 2004 [128]	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	C_{18} Aqua column (150×4.6, 5) gradient elution MP A: 90 % 5 mmol L^{-1} formic acid MP B: 10 % acetonitrile (5 min)	GHB-d6	0.1–10	HPLC–MS–MS APCI(−) injection volume: 10 µL
Johansen and Windberg, 2011 [102]	GHB GBL 1,4-BD GVL	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 C_{18} (150×2.1, 3.5) gradient elution MP A: acidic water MP B: acidic methanol (19 min)	GHB-d6	1–100 mg kg^{-1} blood	HPLC–MS–MS ESI(+) injection volume : 5 µL
Kaufmann and Alt, 2007 [127]	GHB	Urine Serum (250 µL)	LLE: 1) 125 µL 0.1 mol L^{-1} HCl+1 mL ethyl acetate 2) 750 µL *t*-butyl methyl ether Derivatization: 50 µL 3 mol L^{-1} HCl *n*-butanol 50 °C 5 min	C_{18} Zorbax SB-18 Agilent (30×2.1, 3.5) gradient elution MP A: 5 mmol L^{-1} ammonium formate in water MP B: 5 mmol L^{-1} ammonium formate in acetonitrile (NR)	GHB-d6	2–100	HPLC–MS–MS Ion trap injection volume: 10 µL
Lott et al., 2012 [114]	GHB	Serum (100 µL)	PP: 200 µL acetonitrile	Nucleodur HILIC column (NS, 3) isocratic elution MP: 80 % acetonitrile	GHB-d6	2–50	HILIC, HPLC–MS–MS APCI(−) injection volume: 10 µL

Table 1 (continued)

				20 % water with 5 mmol L^{-1} ammonium acetate (15 min)			
Sørensen and Hasselstrøm, 2012 [109]	GHB GBL 1,4-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 mol L^{-1} sodium dihydrogen phosphate solution, 1 mL water; sample loading: 600 μL supernatant+250 μL water	SeQuant ZIC HILIC (100×2.1, 5) gradient elution MP A: 1 mmol L^{-1} ammonium acetate MP B: acetonitrile (10 min)	GHB-d6	5–200	HILIC, UHPLC–MS–MS ESI(+) and ESI(−) injection volume: 10 μL
Wood et al., 2004 [34]	GHB GBL 1,4-BD	Urine (250 μL)	Dilution: 1:20 with deionized water	Atlantis C_{18} column (100×3, 5) isocratic elution MP: 0.1 % aqueous formic acid: methanol (90:10) (11 min)	GHB-d6	1–80	HPLC–MS–MS ESI(+) injection volume: 20 μL
Zacharis et al., 2008 [88]	GHB	Oral fluid (500 μL)	Saliva sample evaporated; residue reconstituted 200 μL DMF (water removal using preheated molecular sieves) Derivatization: 1) 100 μL Br-MMC 70 °C 70 min 2) 100 μL anthracene+1 mL acetonitrile	C_{18} Kromasil (250×4.6, 4) gradient elution MP A: phosphate buffer (40 mmol L^{-1}, pH 3) MP B: methanol (25 min)	anthracene	0.25–7.5	HPLC-Fluorescence detection (330 (λ_{ex}) -390 (λ_{em}) nm) injection volume: 25 μL

Other confirmation method

Ref.	Analyte	Matrix	Extraction	Separation buffer	Calibration Range GHB μg mL^{-1}	Remarks
Gong et al., 2008 [103]	GHB BHB AHB	Urine Serum	Dilution: 1:4 with separation buffer	20 mmol L^{-1} arginine; 10 mmol L^{-1} maleic acid; 30 μmol L^{-1} cetyltrimethylammonium bromide 5 mmol L^{-1} vancomycin	2–400 (urine) 5–150 (serum)	Capillary electrophoresis with contactless conductivity detection

1,4-BD: 1,4-butanediol; AHB: alpha-hydroxybutyric acid; APCI: atmospheric pressure chemical ionization; BHB: beta-hydroxybutyric acid; Br-MMC: 4-bromomethyl-7-methoxycoumarin; BSTFA: *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; CW/TPR: carbowax/templated resin; DMF: dimethylformamide; DMS: dimethyl sulfate; 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*-methyltrifluoroacetamide; NaCl: sodium chloride; NaOH: sodium hydroxide; Na_2SO_4: anhydrous sodium sulfate; 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: tert-butyldimethylchlorosilane; TFAA: trifluoroacetic acid anhydride; TMCS: trimethylchlorosilane; UHPLC: ultra-high performance liquid chromatography; UV: ultraviolet

cartridges). Although an approximately twofold increase in sensitivity was obtained by use of the SPE cartridges, the authors were still able to use the simpler dilution method, because it readily enabled measurement of endogenous GHB levels.

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

In addition, samples have been diluted to reduce the effect 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 have been diluted with water before LLE [105, 106] or SPE [99, 107–109]. For example, Elian et al. [107] assumed that a 50-μL sample and synthetic urine, consisting of inorganic salts and proteins in an aqueous medium, would act similarly to 50 μL deionized water if these were all diluted in 4 mL water before 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.

Deproteinization of the biological fluid

For such compounds as GHB with low protein binding, protein precipitation is an adequate and easy technique for removal of a variety of interferences present in blood and plasma, for example blood cells, proteins, and lipids, before analysis [32, 99]. To illustrate, Shima et al. [32] compared protein precipitation with a variety of SPE and LLE techniques for clean-up of urine, and found that protein precipitation led to the highest GHB recovery and cleanest chromatograms. Generally, there are four protein-precipitation techniques: addition of organic solvents, acids, salts, and metal ions [110]. As far as we are aware, only the first three have been used for GHB analysis.

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

Organic solvents and acids have also been used simultaneously to improve protein precipitation and, in addition, GHB recovery. For example, addition of sulfuric acid during protein precipitation with 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].

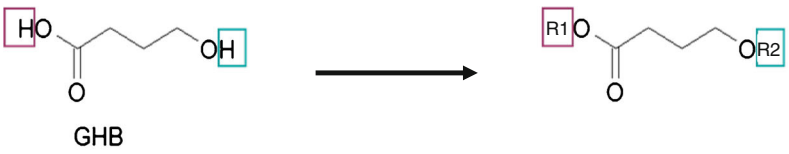
Cold perchloric acid [27, 29, 54] has been added to plasma, thereby combining deproteinization and lactone formation (see below). Finally, anhydrous sodium sulfate (Na_2SO_4) has been added to biological fluids, for protein precipitation and salting-out, before HS-trap analysis [41].

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 before LC–MS–MS analysis. For example, when blood or serum were subjected to protein precipitation, with subsequent centrifugation and 1:1 dilution of the supernatant with acidified water before LC–MS–MS analysis, approximately 40 % suppression of the GHB signal was observed. Again, the importance of using of a deuterated internal standard, for example GHB-d6, is emphasized, because doing so can compensate for the matrix effect [102]. As far as we are aware, only three sample-preparation procedures consist merely of protein precipitation before LC–MS–MS injection, and for only two of these have matrix effects been evaluated [113, 114, 117]. For example, one procedure that did evaluate matrix effects reported 16 to 27 % enhancement of GHB ionization which was compensated for by use of GHB-d6 [117].

Chemical modification of GHB

Because GHB is a polar (sometimes anionic) molecule, with lactone formation occurring at high injector-port temperatures or induced chemically (at low pH), chemical modification before GC is necessary for reliable quantification [75, 118]. For GC analysis, two major strategies have been used: lactone-formation by addition of acid or derivatization by use of a variety of derivatization reagents. Formation of a derivative before LC analysis and detection has also proved useful; however, it has been applied to a lesser extent than before GC-based applications. Both strategies—lactone formation and derivatization—may improve the extraction properties of GHB and/or chromatographic analysis, as discussed in more detail below. Figure 2 gives a schematic overview of possible derivatization procedures, with the resulting derivatives of GHB, using common derivatization reagents.

Chemical modification to improve extraction GHB undergoes intra-molecular esterification within minutes in an acidic environment [40] and the GBL formed is more easily extracted from biological matrices than GHB. Lactone formation has been accomplished by addition of 6 mol L^{-1} hydrochloric acid (urine [27, 29, 119]), concentrated sulfuric acid [8, 120], 20 % trifluoroacetic acid [121], or 1.6 or 0.8 mol L^{-1} perchloric acid



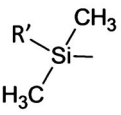
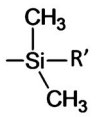
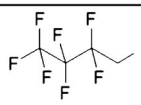
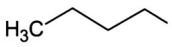
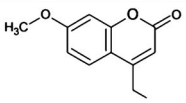
Type of derivatization	R1	R2
Silylation *Reagents:* *R'= -CH_3 e.g. BSTFA, MSTFA* *R'=-$C(CH_3)_3$ e.g. MTBSTFA*		
Methylation *Reagents: e.g. dimethylsulphate*	H_3C-	$-CH_3$
Alkylation/Acetylation *With TFAA/HFB-OH*		
Butylation *With n-butanol*		-H
Alkylchloroformation *Reagents: e.g hexylchloroformate*		-H
To enable fluorescence detection *Reagents: e.g. Br-MMC*		-H

Fig. 2 Overview of derivatization procedures for GHB determination. Br-MMC: 4-bromomethyl-7-methoxy coumarin; BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide; HFB-OH: heptafluorobutanol; MSTFA: N-methyl-N-(trimethylsilyl)-trifluoroacetamide; MTBSTFA: N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide; TFAA: trifluoroacetic acid anhydride

(plasma [27, 29]) to plasma, urine or oral fluid. If perchloric acid is added to plasma, lactone formation and deproteinization are performed in a single step. Subsequently, the acidified sample, or the supernatant after centrifugation [27, 29] are left 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 enable complete conversion. As a consequence, differentiation between the initial GBL present in the sample and the GBL formed as a result of 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 enabling analysis of the samples by solid-phase micro extraction (SPME) or HS-based extraction (and injection) techniques by forming a more volatile derivative of GHB. Hexyl chloroformate, in the presence of pyridine as catalyst, has been used to derivatize the carboxyl group of GHB [38, 104], and dimethyl sulfate (DMS) in alkaline medium has been used to modify both functional groups [41, 95].

To overcome difficulties encountered 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 and organic solvent waste are reduced. Van hee et al. [36] were the first to report extractionless sample preparation, based on direct derivatization of GHB in biofluids with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Addition of excess derivatization reagent to a 20-μL sample of biofluid (serum, plasma, urine) resulted in a simple and rapid method with sufficient sensitivity for routine toxicological analysis. Similarly, starting from 1 μL oral fluid, an extractionless procedure with direct derivatization has been reported, enabling determination of exogenous GHB concentrations [69]. Furthermore, GHB has been derivatized directly ("on spot") in DBS with a mixture of trifluoroacetic acid (TFAA) and heptafluorobutanol (HFB-OH), thereby enabling omission of the extraction step [44, 67].

Chemical modification to improve chromatographic analysis and detection Apart from improving or facilitating extraction, chemical modification may also improve chromatographic analysis and detection. The GC properties of GHB are improved by conversion to the more volatile and stable lactone-form of GBL, achieved by using the same procedures as to improve extraction via GBL formation (see above). Second, a variety of derivatization reagents have been used to increase its molecular weight, at the same time reducing 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 [2, 5, 16, 31–33, 35, 36, 43, 49, 50, 59, 60, 62, 66, 69, 91, 111, 112, 122–124, 126] has been applied, then *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) [105, 115, 125] and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) [37, 106]. By use of these reagents, the hydroxyl and carboxyl functional groups of GHB are derivatized simultaneously, and lactone formation is avoided because acidic conditions are not used [32]. Moreover, to avoid GBL formation and GHB losses during evaporation, Kimura et al. [91] made urine alkaline before derivatization, producing the non-volatile salt form of GHB. Furthermore, the resulting di-trimethylsilyl derivative 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 microwave assisted silylation [35] can be valuable alternatives which reduce analysis 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 used in LC-based separations. For example, to enable fluorescence detection (FD) the carboxyl group has been derivatized by adding 4-bromomethyl-7-methoxycoumarin (Br-MMC) to a water-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 carboxyl function of GHB by use of HCl–*n*-butanol improved detection by ion-trap mass spectrometry [127].

Liquid-liquid extraction (LLE) of GHB or GBL

Liquid–liquid extraction of GHB Solvents commonly used to extract GHB from biological fluids include ethyl acetate [2, 49, 50, 59, 60, 62, 106, 122–127], *t*-butyl methyl ether [105, 127], and hexane [43]. Because the physical properties of GHB make it a poor candidate for LLE, a variety of approaches have been used to enhance transfer of the GHB to the organic solvent (hence increasing its recovery). GHB must be in the uncharged, i.e. neutral, form for optimum extraction yield and selectivity, which are affected by the choice of solvent, pH, and additives [101]. Therefore, the charge on the carboxylic group (pK_a 4.6–4.8) has been influenced by addition of 0.1 mol L^{-1} HCl or cold 0.05 mol L^{-1} H_2SO_4 to urine, serum, and blood, which enhances its transfer to ethyl acetate [59, 122, 127]. Also, for whole-blood, Pan et al. [37] reported the use of a water-scavenging material, for example 80:20 dimethoxypropane–*N*,*N*-dimethylformamide, to facilitate GHB extraction. Salting-out, to increase the ionic strength of the aqueous phase, improving partitioning of relatively water-soluble analytes between two immiscible phases, has also been reported [105]. For this purpose, saturated salt solutions, for example saturated ammonium chloride buffer [51, 124, 126], have been added to the test tubes or NaCl (solid salt) has been added before extraction [105].

Liquid–liquid extraction of GBL After lactone formation (see above), GBL has been extracted from biologic fluids with dichloromethane [8], chloroform [119, 121], or benzene [27], solvents preferably avoided in the modern laboratory. Because GBL may be protonated under the acidic conditions required for complete conversion, recovery 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) by addition of, e.g., phosphate buffer or sodium hydroxide [27, 121]. After LLE, the mixture is usually centrifuged and the supernatant subsequently concentrated, but not completely evaporated, because 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 a low temperatures (max 35 °C) to avoid unacceptable losses of GBL [27].

Solid phase extraction (SPE) of GHB

The first type of SPE sorbent used to extract GHB from biofluids was (strong) anion exchange. When using this type of cartridge, the classical SPE procedure of conditioning, loading, washing, drying, and eluting has been followed. 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, enabling pH-dependent interaction with GHB. At neutral pH, the carboxyl group will be negatively charged (pK_a 4.6–4.8), and will interact with the positively charged sorbent. To elute GHB, it is necessary to neutralize its negative charge by use of an acidic elution solvent [107, 108, 128].

In addition, SPE cartridges can also be used to retain interfering substances, enabling the analyte of interest to pass through the sorbent and be collected for further analysis. For this purpose, Clean Screen SPE cartridges have been used to clean vitreous humor, blood, and urine. The collected eluate contained GHB without substances that could interfere during subsequent analysis [31, 43, 125]. In addition, a (strong) cation-exchange sorbent can be used for clean-up of whole blood after 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, automated SPE (Oasis HLB 30) using a 96-well plate has recently been used for extraction of GHB from whole blood after protein precipitation [99]. Combining this automated SPE with LC–MS–MS resulted in a high-throughput method suitable for screening more than 6,000 samples a year [99].

Also SPME, as a modification of the more classical SPE, has been introduced. In contrast with conventional extraction methods, which use multi-step techniques and excess organic solvents, SPME consists of one solvent-free step only for concentration of the analytes of interest. This technique has been used to determine GHB (derivatized with hexyl chloroformate) in urine, by use of a fused-silica fiber coated with a stationary phase which adsorbed the analytes of interest. The SPME fiber can be placed directly in the sample or, alternatively, in the headspace [38, 104]. (Headspace extraction of GHB is discussed below.)

Headspace extraction of GBL or derivatized GHB

Few of the GC methods presented use HS as extraction and injection technique. The reason may be not only the more complex optimization of these procedures but also the fact that, typically, a larger sample volume is required to achieve sensitivity similar to that of more traditional sample preparation procedures, for example LLE or SPE [29, 104]. Also the requirement for a more specific configuration, which may also imply use of a more specific analytical column (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 fewer 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 sample, anhydrous salt (to enhance transfer of the analyte of interest to the headspace, thus increasing its recovery), and derivatization reagent or acid for lactone formation to an HS vial. After appropriate sealing, the vial can be placed in the HS oven for analysis.

Headspace extraction of derivatized GHB Combining "in-vial" derivatization with headspace injection techniques may extend the range of application normally reserved for volatile compounds to semi-volatile or non-volatile analytes such as GHB. After derivatization with hexyl chloroformate or dimethyl sulfate, derivatized GHB has been extracted by SPME or HS-trap, respectively [38, 41, 95]. Both methods have little sample-preparation time. The method using SPME is one of the most sensitive methods reported, having an LLOQ of 0.1 μg mL^{-1}, starting from 0.5 mL urine. The HS-trap method is suitable for determination of GHB in a variety of biological fluids, requiring only 100 μL sample.

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

Chromatographic analysis and detection

Gas chromatography

Although the nature of GHB does not favor use of GC (see above), it remains the most popular separation technique of the last two decades, enabled by use of appropriate sample preparation techniques. Toxicological analysis is commonly performed with an analytical column containing a polysiloxane stationary phase with 95 % methyl groups and 5 % phenyl groups, which is also well suited for determination of derivatized GHB and/or GBL (and analogues), reflected in its wide use. Most GC-based methods focus on the detection of GHB, either in the derivatized form or as GBL; a few methods also include simultaneous analysis of glycols, BHB, GHV, 1,4-BD, and/or SSA [2, 31, 35, 36, 39, 123].

Gas chromatography–flame ionization detection Although this universal detector has been used for initial screening for GHB, followed by confirmation using GC–MS [8, 56], Jones et al. [116] used GC–flame ionization detection (FID) to quantify GHB as GBL in blood within a wide calibration range, starting at 8 μg mL^{-1}.

Gas chromatography–mass spectrometry To unequivocally identify and quantify GHB in biological fluids, GC is preferably used in conjunction with mass spectrometry. It has been used with electron impact (EI) ionization and in positive or negative chemical-ionization modes (PICI or NICI). For quantification, the MS operates in SIM (selected ion monitoring) mode, in which

ions of *m/z* typical of GBL or derivatized GHB are monitored. Derivatization using silylating or other derivatizing reagents, to increase the molecular weight and the fragments' masses, is generally advantageous for MS detection, because 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 [59] and Chen et al. [111] to quantify GHB in biofluids after silylation, and by Lenz et al. [120] and Frison et al. [29] after conversion of GHB to GBL. Although one method had a lower LLOQ of 0.4 μg mL^{-1}, no relevant gain in sensitivity was observed compared with GC methods in which the MS is used in EI mode. On the other hand, using the MS in NICI mode to quantify GHB as GBL in plasma has been shown suitable for determination of endogenous concentrations, with a calibration range situated in the low μg mL^{-1} range [119].

Although not routinely performed using GC-based methods, simultaneous analysis of GHB and 1,4-BD or other compounds, for example BHB and SSA, is possible, as was done by Lora-Tamayo et al. and Sakurada et al., [2, 123] respectively, who only slightly modified the method of Couper and Logan [122]. GHV and GHB can be analyzed simultaneously [31], and Andresen-Streichert et al. [39] recently reported a GC–MS method for simultaneous analysis of GHB and GHV in urine, with an extraction and derivatization procedure based on the method published by Kerrigan [59].

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. 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 is not crucial for blood and urine, because GHB is endogenously present at relatively high concentrations (sub and low microgram-per-milliliter range). Nonetheless, MS–MS may still result in improved peak shape, which is required for reliable integration [33]. Although tandem MS may have the advantage over existing methods of providing spectra free from background contaminants, and, thus, of being more selective, it remains or becomes even more important to evaluate whether the di-TMS derivative of GHB is free from interferences from compounds with the same precursor ion (*m/z* 233), for example its positional isomers, AHB and BHB [33].

Liquid chromatography

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

Liquid chromatography with ultraviolet or fluorescence detection Because GHB has no chromophoric group, UV-detection is only possible at low wavelength (220 nm), as reported by De Vriendt et al. [108]. Starting from 60 μL plasma, quantification was possible in the range 10 to 750 μg mL^{-1}, the LLOQ being 5 to 10-fold higher than for most of the confirmatory methods reported here. Introducing an UV-active or fluorescent group by derivatization should result in enhanced sensitivity and improved certainty of identification, as illustrated by Zacharis et al. [89]. These authors, starting from 500 μL oral fluid, derivatized GHB, producing a highly fluorescent derivative, with the lowest calibrator corresponding to 0.25 μg mL^{-1}.

Liquid chromatography–tandem mass spectrometry UHPLC–MS–MS has the potential for shorter run times and improved sensitivity and precision compared with more traditional separation methods, for example HPLC–UV or HPLC–FL; this is also facilitated by the possibility of using a stable isotopically labeled internal standard. For example, Fung et al. [128] 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 0.1 μg mL^{-1}).

Also, LC–MS–MS may enable simultaneous analysis of GHB and its precursors, GBL and 1,4-BD [34], by use of 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, because, under some conditions in ESI(+), the molecule might lose water

within the instrument source with formation of GBL, it is of interest that the method can distinguish between in-source generated GBL or $[GHB-H_2O]^+$ and actual GBL in a sample [34, 102, 109]. It is interesting to note that in one method [102] this in-source conversion of GHB to GBL resulted in sufficient sensitivity for determination of GHB in whole blood whereas others [34, 109] achieved relatively low (6 %) conversion, which was unsuitable for GHB quantification.

Alternatively, to counter the detection of small *m*/*z* ions typical of GHB (*m*/*z* parent ion=103), an LC–MS–MS method for GHB in human serum has recently been reported in which quantification was based on fragmentation of adducts formed with components of the mobile phase, more specifically fragmentation of the GHB–sodium acetate adduct in ESI(−) mode (*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 C_{18} columns frequently used for GHB separation require acidified mobile phases for better control of the retention of GHB (which, being a weak acid with a pK_a of 4.6, is uncharged in acidic mobile phases only) [99]. This may result in restriction to ESI(+) mode only, because the acidic conditions used may reduce the response to GHB in ESI(−) mode [99, 109]. However, Forni et al. reported lower background noise under their chromatographic conditions with the MS–MS operating in ESI(−) as compared with ESI(+) [68].

Sørensen et al. [109] and Lott et al. [114] suggested use of hydrophilic interaction liquid chromatography (HILIC) to overcome this problem and to improve retention and chromatographic separation of small and polar molecules. HILIC enables chromatography to be performed under neutral conditions, optimal for separation of GHB and its analogues and which also prevents inter-conversion between GHB and GBL [109]. Thus, when a typical C_{18} reversed-phase column is used, GHB elutes first, followed by 1,4-BD and GBL, whereas when HILIC is used the order of elution 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 the cost of analysis by high-throughput 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, for example instrumental back-up, may affect the choice between RP and HILIC [99].

Non-chromatographic techniques

Gong et al. [103] reported a CZE–C^4D method for analysis of GHB in urine and serum. Although not commonly used for toxicological analysis, this technique is well-suited to determine small ions, for example GHB in the anionic form [118]. Separation and detection of AHB, BHB, and GHB were achieved without preceding extraction or derivatization, merely simple 1:4 dilution with an optimized separation buffer of pH>4.7 to deprotonate the analytes and convert them to the anions. In addition, the more alkaline pH also inhibits conversion of GHB to GBL. The sensitivity of the method was sufficient for discrimination between endogenous and exogenous GHB levels in urine (cut-off 10 μg mL^{-1}). Also, the instrumentation is less expensive than that used for the other techniques used in clinical and forensic laboratories, and a portable instrument may enable on-site analysis of urine samples from a suspected GHB intoxication [103].

Conclusion

To conclude, a variety of screening and confirmation methods are available for analysis of GHB (and analogues if required) in biological fluids. GHB screening and analysis in a toxicological laboratory are mostly performed because of suspected ingestion of this club drug, supported by information from the police or physician, rather than on a routine basis, as is the case for more widely abused drugs, for example cannabinoids, amphetamines, and opioids [54, 116]. However, routine screening has become possible, not only as a result of the commercial availability of an enzymatic kit for GHB suitable for common chemical analyzers but also because of the availability of simpler GC–MS methods and more sophisticated techniques, for example UHPLC–MS–MS, which—when combined with automated sample-preparation procedures—enable 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, which necessitates conversion to GBL or derivatization to a more volatile and stable form. However, LC-based applications coupled to tandem MS are increasingly attracting interest, because they have the advantage of simpler sample-preparation (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 (for example from GBL formed in the source during MS–MS analysis) [33]. With regard to method sensitivity, GC and LC-based applications have similar LLOQs, but, as Kankaanpää et al. fittingly remarked "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, when results have been obtained by use of the screening and confirmation methods discussed above, interpretation is a second challenge for the toxicologist, and analysis of different matrices may be useful for correct interpretation. For example, Kintz et al. [52] suggested analysis of several specimens, including different types of blood and vitreous humor, in the case of a suspected GHB-related death. On the other hand, the question arises whether or not analysis of different matrices, for example oral fluid, sweat, and hair, has the potential to extend detection sensitivity and/or furnish results of more value than those obtained from analysis of 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.

References

1. Neijzen R, van Ardenne P, Sikma M, Egas A, Ververs T, van Maarseveen E (2012) Activated charcoal for GHB intoxication: an in vitro study. Eur J Pharm Sci 47(5):801–803
2. Sakurada K, Kobayashi M, Iwase H, Yoshino M, Mukoyama H, Takatori T, Yoshida K (2002) Production of gamma-hydroxybutyric acid in postmortem liver increases with time after death. Toxicol Lett 129(3):207–217
3. Thai D, Dyer JE, Jacob P, Haller CA (2007) Clinical pharmacology of 1,4-butanediol and gamma-hydroxybutyrate after oral 1,4-butanediol administration to healthy volunteers. Clin Pharmacol Ther 81(2):178–184
4. Smith KM, Larive LL, Romanelli F (2002) Club drugs: methylenedioxymethamphetamine, flunitrazepam, ketamine hydrochloride, and gamma-hydroxybutyrate. Am J Health Syst Pharm 59(11):1067–1076
5. Abanades S, Farre M, Segura M, Pichini S, Barral D, Pacifici R, Pellegrini M, Fonseca F, Langohr K, De La Torre R (2006) Gamma-hydroxybutyrate (GHB) in humans: Pharmacodynamics and pharmacokinetics. Ann N Y Acad Sci 1074:559–576
6. Miotto K, Darakjian J, Basch J, Murray S, Zogg J, Rawson R (2001) Gamma-hydroxybutyric acid: Patterns of use, effects and withdrawal. Am J Addict 10(3):232–241
7. Carter LP, Pardi D, Gorsline J, Griffiths RR (2009) Illicit gamma-hydroxybutyrate (GHB) and pharmaceutical sodium oxybate (Xyrem): differences in characteristics and misuse. Drug Alcohol Depend 104(1–2):1–10
8. LeBeau MA, Montgomery MA, Miller ML, Burmeister SG (2000) Analysis of biofluids for gamma-hydroxybutyrate (GHB) and gamma-butyrolactone (GBL) by headspace GC–FID and GC–MS. J Anal Toxicol 24(6):421–428
9. LeBeau MA, Montgomery MA, Morris-Kukoski C, Schaff JE, Deakin A (2007) Further evidence of in vitro production of gamma-hydroxybutyrate (GHB) in urine samples. Forensic Sci Int 169(2–3):152–156
10. ElSohly MA, Salamone SJ (1999) Prevalence of drugs used in cases of alleged sexual assault. J Anal Tox 23(3):141–146
11. Zvosec DL, Smith SW, Porrata T, Strobl AQ, Dyer JE (2011) Case series of 226 γ-hydroxybutyrate–associated deaths: lethal toxicity and trauma. Am J Emerg Med 29(3):319–332
12. Li J, Stokes SA, Woeckener A (1998) A tale of novel intoxication: seven cases of gamma-hydroxybutyric acid overdose. Ann Emerg Med 31(6):723–728
13. Andresen H, Stimpfl T, Sprys N, Schnitgerhans T, Müller A (2011) Liquid ecstasy - a significant drug problem. Dtsch Arztebl Int 105(36):599–603
14. Anderson IB, Kim SY, Dyer JE, Burkhardt CB, Iknoian JC, Walsh MJ, Blanc PD (2006) Trends in gamma-hydroxybutyrate (GHB) and related drug intoxication: 1999 to 2003. Ann Emerg Med 47(2): 177–183
15. EMCDDA website: http://www.emcdda.europa.eu/publications/edr/trends-developments/2013. Accessed 30 Oct 2013
16. Villain M, Cirimele V, Ludes B, Kintz P (2003) Ultra-rapid procedure to test for gamma-hydroxybutyric acid in blood and urine by gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 792(1):83–87
17. Van Amsterdam JGC, Brunt TM, McMaster MTB, Niesink RJM (2012) Possible long-term effects of γ-hydroxybutyric acid (GHB) due to neurotoxicity and overdose. Neurosci Biobehav Rev 36(4): 1217–1227
18. Schep LJ, Knudsen K, Slaughter RJ, Vale JA, Mégarbane B (2012) The clinical toxicology of γ-hydroxybutyrate, γ-butyrolactone and 1,4-butanediol. Clin Toxicol 50(6):458–470
19. Wood DM, Nicolaou M, Dargan PI (2009) Epidemiology of recreational drug toxicity in a nightclub environment. Subst Use Misuse 44:1495–1502
20. Van Sassenbroeck DK, De Neve N, De Paepe P, Belpaire FM, Verstraete AG, Calle PA, Buylaert WA (2007) Abrupt awakening phenomenon associated with gamma-hydroxybutyrate use: a case series. Clin Toxicol 45(5):533–538
21. Dyer JE, Roth B, Hyma BA (2001) Gamma-hydroxybutyrate withdrawal syndrome. Ann Emerg Med 37(2):147–153
22. Wojtowicz JM, Yarema MC, Wax PM (2008) Withdrawal from gamma-hydroxybutyrate, 1,4-butanediol and gamma-butyrolactone: a case report and systematic review. CJEM 10(1): 69–74
23. Doherty JD, Hattox SE, Snead OC, Roth RH (1978) Identification of endogenous gamma-hydroxybutyrate in human and bovine brain and its regional distribution in human, guinea pig and rhesus monkey brain. J Pharmacol Exp Ther 207(1):130–139
24. Lettieri J, Fung HL (1978) Improved pharmacological activity via pro-drug modification: comparative pharmacokinetics of sodium gamma-hydroxybutyrate and gamma-butyrolactone. Res Commun Chem Pathol Pharmacol 22(1):107–118
25. Vree TB, Van der Kleijn E, Knop HJ (1976) Rapid determination of 4-hydroxybutyric acid and 2-propylpentanoate in human plasma by means of gas-liquid chromatography. J Chrom 121:150–152
26. Hoes MJ, Vree TB, Guelen PJ (1981) Circadian rhythm in plasma concentrations of gamma-hydroxybutyric acid in alcoholics. Int J Addict 16(6):1071–1075
27. Ferrara SD, Tedeschi L, Frison G, Castagna F, Gallimberti L, Giorgetti R, Gessa GL, Palatini P (1993) Therapeutic gamma-hydroxybutyric acid monitoring in plasma and urine by gas chromatography-mass spectrometry. J Pharm Biomed Anal 11(6): 483–487
28. Maurer HH (2004) Position of chromatographic techniques in screening for detection of drugs or poisons in clinical and forensic toxicology and/or doping control. Clin Chem Lab Med 42(11): 1310–1324
29. Frison G, Tedeschi L, Maietti S, Ferrara SD (2000) Determination of γ-hydroxybutyric acid (GHB) in plasma and urine by headspace solid-phase microextraction and gas chromatography/positive ion chemical ionization mass spectrometry. Rapid Com Mass Spectr 14(24):2401–2407
30. Lenz D, Rothschild MA, Kröner L (2008) Intoxications due to ingestion of gamma-butyrolactone: organ distribution of gamma-hydroxybutyric acid and gamma-butyrolactone. Ther Drug Monit 30(6):755–761

31. Marinetti LJ, Isenschmid DS, Hepler BR, Kanluen S (2005) Analysis of GHB and 4-methyl-GHB in postmortem matrices after long-term storage. J Anal Toxicol 29(1):41–47
32. Shima N, Miki A, Kamata T, Katagi M, Tsuchihashi H (2005) Endogenous level and in vitro production of GHB in blood from healthy humans, and the interpretation of GHB levels detected in antemortem blood samples. J Health Sci 51(2):147–154
33. Paul R, Tsanaclis L, Kingston R, Berry A, Guwy A (2006) GC–MS-MS determination of gamma-hydroxybutyrate in blood and urine. J Anal Toxicol 30(6):375–379
34. Wood M, Laloup M, Samyn N, Morris MR, de Bruijn EA, Maes RA, Young MS, Maes V, De Boeck G (2004) Simultaneous analysis of gamma-hydroxybutyric acid and its precursors in urine using liquid chromatography-tandem mass spectrometry. J Chromatogr A 1056(1–2):83–90
35. Meyer MR, Weber AA, Maurer HH (2011) A validated GC–MS procedure for fast, simple, and cost-effective quantification of glycols and GHB in human plasma and their identification in urine and plasma developed for emergency toxicology. Anal Bioanal Chem 400(2):411–414
36. Van hee P, Neels H, De Doncker M, Vrydags N, Schatteman K, Uyttenbroeck W, Hamers N, Himpe D, Lambert WE (2004) Analysis of gamma-hydroxybutyric acid, DL-lactic acid, glycolic acid, ethylene glycol and other glycols in body fluids by a direct injection gas chromatography-mass spectrometry assay for wide use. Clin Chem Lab Med 42(11):1341–1345
37. Pan YM, Gill GN, Tilson CS, Wall WH, McCurdy HH (2001) Improved procedure for the analysis of gamma-hydroxybutyrate and ethylene glycol in whole blood. J Anal Toxicol 25(5):328–332
38. Brown SD, Rhodes DJ, Pritchard BJ (2007) A validated SPME-GC–MS method for simultaneous quantification of club drugs in human urine. Forensic Sci Int 171(2–3):142–150
39. Andresen-Streichert H, Jungen H, Gehl A, Müller A, Iwersen-Bergmann S (2013) Uptake of gamma-valerolactone–detection of gamma-hydroxyvaleric acid in human urine samples. J Anal Toxicol 37(4):250–254
40. Ciolini LA, Mesmer MZ, Satzger RD, Machal AC, McCauley HA, Mohrhaus AS (2001) The chemical interconversion of GHB and GBL: forensic issues and implications. J Forensic Sci 46:1315–1323
41. Ingels ASME, Neels H, Lambert WE, Stove CP (2013) Determination of gamma-hydroxybutyric acid in biofluids using a one-step procedure with "in-vial" derivatization and headspace-trap gas chromatography-mass spectrometry. J Chromatogr A 1296:84–92
42. Blanchet B, Morand K, Hulin A, Astier A (2002) Capillary gas chromatographic determination of 1,4-butanediol and gamma-hydroxybutyrate in human plasma and urine. J Chromatogr B Analyt Technol Biomed Life Sci 769(2):221–226
43. McCusker RR, Paget-Wilkes H, Chronister CW, Goldberger BA (1999) Analysis of gamma-hydroxybutyrate (GHB) in urine by gas chromatography-mass spectrometry. J Anal Toxicol 23(5):301–305
44. Ingels AS, Lambert WE, Stove CP (2010) Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC–MS method with direct "on spot" derivatization. Anal Bioanal Chem 398(5):2173–2182
45. Elliott SP (2004) Further evidence for the presence of GHB in postmortem biological fluid: implications for the interpretation of findings. J Anal Toxicol 28(1):20–26
46. LeBeau MA, Miller ML, Levine B (2001) Effect of storage temperature on endogenous GHB levels in urine. Forensic Sci Int 119(2):161–167
47. LeBeau MA, Christenson RH, Levine B, Darwin WD, Huestis MA (2002) Intra- and interindividual variations in urinary concentrations of endogenous gamma-hydroxybutyrate. J Anal Toxicol 26(6):340–346
48. Elian AA (2002) Determination of endogenous gamma-hydroxybutyric acid (GHB) levels in antemortem urine and blood. Forensic Sci Int 128(3):120–122
49. Elliott SP (2003) Gamma hydroxybutyric acid (GHB) concentrations in humans and factors affecting endogenous production. Forensic Sci Int 133(1–2):9–16
50. Andresen H, Sprys N, Schmoldt A, Mueller A, Iwersen-Bergmann S (2010) Gamma-hydroxybutyrate in urine and serum: Additional data supporting current cut-off recommendations. Forensic Sci Int 200(1–3):93–99
51. De Paoli G, Walker KM, Pounder DJ (2011) Endogenous γ-hydroxybutyric acid concentrations in saliva determined by gas chromatography-mass spectrometry. J Anal Toxicol 35(3):148–152
52. Kintz P, Villain M, Cirimele V, Ludes B (2004) GHB in postmortem toxicology. Discrimination between endogenous production from exposure using multiple specimens. Forensic Sci Int 143(2–3):177–181
53. Ingels M, Rangan C, Bellezzo J, Clark RF (2000) Coma and respiratory depression following the ingestion of ghb and its precursors: Three cases. J Emerg Med 19(1):47–50
54. Bosman IJ, Lusthof KJ (2003) Forensic cases involving the use of GHB in the Netherlands. Forensic Sci Int 133(1–2):17–21
55. Kintz P, Villain M, Pelissier AL, Cirimele V, Leonetti G (2005) Unusually high concentrations in a fatal GHB case. J Anal Toxicol 29(6):582–585
56. Elliott SP (2004) Nonfatal instances of intoxication with gamma-hydroxybutyrate in the United Kingdom. Ther Drug Monit 26(4):432–440
57. Morris-Kukoski CL (2004) γ-Hydroxybutyrate. Bridging the clinical-analytical gap. Toxicol Rev 23(1):33–43
58. TIAFT website; http://www.tiaft.org/. Accessed 9 Sept 2013
59. Kerrigan S (2002) In vitro production of gamma-hydroxybutyrate in antemortem urine samples. J Anal Toxicol 26(8):571–574
60. Elliott S, Lowe P, Symonds A (2004) The possible influence of micro-organisms and putrefaction in the production of GHB in postmortem biological fluid. Forensic Sci Int 139(2–3):183–190
61. Kugelberg FC, Holmgren A, Eklund A, Jones AW (2010) Forensic toxicology findings in deaths involving gamma-hydroxybutyrate. Int J Legal Med 124(1):1–6
62. Crookes CE, Faulds MC, Forrest AR, Galloway JH (2004) A reference range for endogenous gamma-hydroxybutyrate in urine by gas chromatography-mass spectrometry. J Anal Toxicol 28(8):644–649
63. Ingels AS, Hertegonne KB, Lambert WE, Stove CP (2013) Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home: an exploratory study. CNS Drugs 27(3):233–237
64. Saudan C, Augsburger M, Kintz P, Saugy M, Mangin P (2005) Detection of exogenous GHB in blood by gas chromatography-combustion-isotope ratio mass spectrometry: implications in postmortem toxicology. J Anal Toxicol 29(8):777–781
65. Palatini P, Tedeschi L, Frison G, Padrini R, Zordan R, Orlando R, Gallimberti L, Gessa GL, Ferrara SD (1993) Dose-dependent absorption and elimination of gamma-hydroxybutyric acid in healthy volunteers. Eur J Clin Pharmacol 45(4):353–356
66. Abanades S, Farré M, Segura M, Pichini S, Pastor A, Pacifici R, Pellegrini M, de la Torre R (2007) Disposition of gamma-hydroxybutyric acid in conventional and nonconventional biologic fluids after single drug administration: issues in methodology and drug monitoring. Ther Drug Monit 29(1):64–70
67. Ingels AS, De Paepe P, Anseeuw K, Van Sassenbroeck DK, Neels H, Lambert WE, Stove CP (2011) Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid

intoxications: validation of an optimized GC–MS procedure. Bioanalysis 3:2271–2281
68. Forni S, Pearl PL, Gibson KM, Yu Y, Sweetman L (2013) Quantitation of gamma-hydroxybutyric acid in dried blood spots: feasibility assessment for newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency. Mol Genet Metab 109(3):255–259
69. De Paoli G, Bell S (2008) A rapid GC–MS determination of gamma-hydroxybutyrate in saliva. J Anal Toxicol 32(4):298–302
70. Kintz P, Goullé JP, Cirimele V, Ludes B (2001) Window of Detection of γ-Hydroxybutyrate in Blood and Saliva. Clin Chem 47(11):2033–2034
71. Kintz P, Cirimele V, Jamey C, Ludes B (2003) Testing for GHB in hair by GC/MS/MS after a single exposure. Application to document sexual assault. J Forensic Sci 48(1):195–200
72. Goullé JP, Chèze M, Pépin G (2003) Determination of endogenous levels of GHB in human hair. Are there possibilities for the identification of GHB administration through hair analysis in cases of drug-facilitated sexual assault? J Anal Toxicol 27(8):574–580
73. Cirimele V, Baumgartner M, Vallet E, Duez M (2010) Interpretation of GHB concentrations in hair. Ann Toxicol Analyt 22(4):161–164
74. Petersen IN, Tortzen C, Kristensen JL, Pedersen DS, Breindahl T (2013) Identification of a new metabolite of GHB: gamma-hydroxybutyric acid glucuronide. J Anal Toxicol 37(5):291–297
75. Grootveld M, Algeo D, Silwood CJ, Blackburn JC, Clark AD (2006) Determination of the illicit drug gamma-hydroxybutyrate (GHB) in human saliva and beverages by 1H NMR analysis. Biofactors 27(1–4):121–136
76. Marquet P (2012) LC-MS vs. GC–MS, online extraction systems, advantages of technology for drug screening assays. Methods Mol Biol 902:15–27
77. Bühlmann laboratories, http://www.buhlmannlabs.ch/core/clinical-chemistry/ghb-kinetic/. Accessed 26 Aug 2013
78. Badcock NR, Zotti R (1999) Rapid screening test for gamma-hydroxybutyric acid (GHB, Fantasy) in urine. Ther Drug Monit 21(3):376
79. Alston WC 2nd, Ng K (2002) Rapid colorimetric screening test for gamma-hydroxybutyric acid (liquid X) in human urine. Forensic Sci Int 126(2):114–117
80. Bravo DT, Harris DO, Parsons SM (2004) Reliable, sensitive, rapid and quantitative enzyme-based assay for gamma-hydroxybutyric acid (GHB). J Forensic Sci 49(2):379–387
81. Drugcheck, http://www.drugcheck.com/dc_ghb.html. Accessed 26 Aug 2013
82. Sciotti MA, Hasan L, Scholer A, Jermann TM, Weber JM, Gygax D (2010) Development and characterization of an enzymatic method for the rapid determination of gamma hydroxybutyric acid. Chimia 64(11):793–798
83. Hasan L, Jermann TM, Weber JM, Abrahamsson L, Sciotti MA, Böttcher M, Jöchle W, Gygax D, Scholer A (2011) An enzymatic method to determine γ-hydroxybutyric acid in serum and urine. Ther Drug Monit 33(6):757–765
84. Grenier V, Huppé G, Lamarche M, Mireault P (2012) Enzymatic assay for GHB determination in forensic matrices. J Anal Toxicol 36(7):523–528
85. Del Signore AG, McGregor M, Cho BP (2005) 1H NMR analysis of GHB and GBL: further findings on the interconversion and a preliminary report on the analysis of GHB in serum and urine. J Forensic Sci 50(1):81–86
86. Mercer J, Shakleya D, Bell S (2006) Applications of ion mobility spectrometry (IMS) to the analysis of gamma-hydroxybutyrate and gamma-hydroxyvalerate in toxicological matrices. J Anal Toxicol 30(8):539–544
87. Gottardo R, Bortolotti F, Trettene M, De Paoli G, Tagliaro F (2004) Rapid and direct analysis of gamma-hydroxybutyric acid in urine by capillary electrophoresis-electrospray ionization ion-trap mass spectrometry. J Chromatogr A 1051(1–2):207–211
88. Baldacci A, Theurillat R, Caslavska J, Pardubská H, Brenneisen R, Thormann W (2003) Determination of gamma-hydroxybutyric acid in human urine by capillary electrophoresis with indirect UV detection and confirmation with electrospray ionization ion-trap mass spectrometry. J Chromatogr A 990(1–2):99–110
89. Zacharis CK, Raikos N, Giouvalakis N, Tsoukali-Papadopoulou H, Theodoridis GA (2008) A new method for the HPLC determination of gamma-hydroxybutyric acid (GHB) following derivatization with a coumarin analogue and fluorescence detection: application in the analysis of biological fluids. Talanta 75(2):356–361
90. Bortolotti F, De Paoli G, Gottardo R, Trattene M, Tagliaro F (2004) Determination of gamma-hydroxybutyric acid in biological fluids by using capillary electrophoresis with indirect detection. J Chromatogr B Analyt Technol Biomed Life Sci 800(1–2):239–244
91. Kimura M, Hasegawa Y, Nakagawa K, Kajita M, Watanabe K, Yamaguchi S (2003) A sensitive method for 4-hydroxybutyric acid in urine using gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 792(1):141–144
92. Shinka T, Inoue Y, Ohse M, Ito A, Ohfu M, Hirose S, Kuhara T (2002) Rapid and sensitive detection of urinary 4-hydroxybutyric acid and its related compounds by gas chromatography-mass spectrometry in a patient with succinic semialdehyde dehydrogenase deficiency. J Chromatogr B Analyt Technol Biomed Life Sci 776(1):57–63
93. Quang LS, Levy HL, Law T, Desai MC, Maher TJ, Boyer EW, Shannon MW, Woolf AD (2005) Laboratory diagnosis of 1,4-BD and GHB overdose by routine urine organic acid analysis. Clin Toxicol 43(4):321–323
94. Garg U, Scott D, Kiscoan M, Knoblauch J, Frazee CC 3rd, Wasserman G (2007) Use of urine organic acids method for the analysis of gamma-hydroxybutric acid (GHB): importance of urease in eliminating urea interference. Clin Toxicol 45(2):194–196
95. Rasanen I, Viinamäki J, Vuori E, Ojanperä I (2010) Headspace in-tube extraction gas chromatography-mass spectrometry for the analysis of hydroxylic methyl-derivatized and volatile organic compounds in blood and urine. J Anal Toxicol 34(3): 113–121
96. Adamowicz P, Kała M (2010) Simultaneous screening for and determination of 128 date-rape drugs in urine by gas chromatography-electron ionization-mass spectrometry. Forensic Sci Int 198(1–3):39–45
97. Stove CP, Ingels AS, Lambert WE (2013) Quantitation of gamma-hydroxybutyric acid in dried blood spots in newborns screening. Mol Genet Metab 110(1–2):195
98. Forni S, Pearl PL, Gibson KM, Yu Y, Sweetman L (2013) Response to Stove and colleagues concerning newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency in dried blood spots. Mol Genet Metab 110(1–2):196
99. Dahl SR, Olsen KM, Strand DH (2012) Determination of γ-hydroxybutyrate (GHB), β-hydroxybutyrate (BHB), pregabalin, 1, 4-butane-diol (1,4BD) and γ-butyrolactone (GBL) in whole blood and urine samples by UPLC-MSMS. J Chromatogr B Analyt Technol Biomed Life Sci 885–886:37–42
100. Kraemer T, Paul LD (2007) Bioanalytical procedures for determination of drugs of abuse in blood. Anal Bioanal Chem 388(7):1415–1435
101. Smith RM (2003) Before the injection–modern methods of sample preparation for separation techniques. J Chromatogr A 1000(1–2): 3–27
102. Johansen SS, Windberg CN (2011) Simultaneous determination of γ-Hydroxybutyrate (GHB) and its analogues (GBL, 1.4-BD, GVL) in whole blood and urine by liquid chromatography coupled to tandem mass spectrometry. J Anal Toxicol 35(1):8–14

103. Gong XY, Kubán P, Scholer A, Hauser PC (2008) Determination of gamma-hydroxybutyric acid in clinical samples using capillary electrophoresis with contactless conductivity detection. J Chromatogr A 1213(1):100–104
104. Blair S, Song M, Hall B, Brodbelt J (2001) Determination of gamma-hydroxybutyrate in water and human urine by solid phase microextraction-gas chromatography/quadrupole ion trap spectrometry. J Forensic Sci 46(3):688–693
105. Kankaanpää A, Liukkonen R, Ariniemi K (2007) Determination of gamma-hydroxybutyrate (GHB) and its precursors in blood and urine samples: a salting-out approach. Forensic Sci Int 170(2–3): 133–138
106. Elie MP, Baron MG, Birkett JW (2012) Injection port silylation of γ-hydroxybutyrate and trans-hydroxycrotonic acid: conditions optimisation and characterisation of the di-tert-butyldimethylsilyl derivatives by GC–MS. Analyst 137(1):255–262
107. Elian AA, Hackett J (2011) Anion exchange SPE and liquid chromatography-tandem mass spectrometry in GHB analysis. J Chromatogr B Analyt Technol Biomed Life Sci 879(31):3752–3758
108. de Vriendt CA, van Sassenbroeck DK, Rosseel MT, van de Velde EJ, Verstraete AG, Vander Heyden Y, Belpaire FM (2001) Development and validation of a high-performance liquid chromatographic method for the determination of gamma-hydroxybutyric acid in rat plasma. J Chromatogr B Biomed Sci Appl 752(1):85–90
109. Sørensen LK, Hasselstrøm JB (2012) A hydrophilic interaction liquid chromatography electrospray tandem mass spectrometry method for the simultaneous determination of γ-hydroxybutyrate and its precursors in forensic whole blood. Forensic Sci Int 222(1–3):352–359
110. Polson C, Sarkar P, Incledon B, Raguvaran V, Grant R (2003) Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 785(2):263–275
111. Chen M, Andrenyak DM, Moody DE, Foltz RL (2003) Stability of plasma gamma-hydroxybutyrate determined by gas chromatography-positive ion chemical ionization-mass spectrometry. J Anal Toxicol 27(7):445–458
112. Louagie HK, Verstraete AG, De Soete CJ, Baetens DG, Calle PA (1997) A sudden awakening from a near coma after combined intake of gamma-hydroxybutyric acid (GHB) and ethanol. J Toxicol Clin Toxicol 35(6):591–594
113. Dresen S, Kempf J, Weinmann W (2007) Prevalence of gamma-hydroxybutyrate (GHB) in serum samples of amphetamine, metamphetamine and ecstasy impaired drivers. Forensic Sci Int 173(2–3):112–116
114. Lott S, Musshoff F, Madea B (2012) Estimation of gamma-hydroxybutyric acid (GHB) co-consumption in serum samples of drivers positive for amphetamine or ecstasy. Forensic Sci Int 221(1–3):98–101
115. Duer WC, Byers KL, Martin JV (2001) Application of a convenient extraction procedure to analyze gamma-hydroxybutyric acid in fatalities involving gamma-hydroxybutyric acid, gamma-butyrolactone, and 1,4-butanediol. J Anal Toxicol 25(7):576–582
116. Jones AW, Holmgren A, Kugelberg FC (2007) Gamma-hydroxybutyrate concentrations in the blood of impaired drivers, users of illicit drugs, and medical examiner cases. J Anal Toxicol 31(9):566–572
117. Dziadosz M, Weller JP, Klintschar M, Teske J (2013) Adduct supported analysis of γ-hydroxybutyrate in human serum with LC–MS–MS. Anal Bioanal Chem 405(20):6595–6597
118. Van Bocxlaer JF (2005) Recent trends in analytical procedures in forensic toxicology. Ther Drug Monit 27(6):752–755
119. Fukui Y, Matsusima E, Muramoto K, Nagai N, Ohama K, Yamashita K (2003) Validation of a simple gas chromatographic-mass spectrometric method for the determination of gamma-butyrolactone in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 785(1):73–80
120. Lenz D, Kröner L, Rothschild MA (2009) Determination of gamma-hydroxybutyric acid in serum and urine by headspace solid-phase dynamic extraction combined with gas chromatography-positive chemical ionization mass spectrometry. J Chromatogr A 1216(18): 4090–4096
121. Brenneisen R, Elsohly MA, Murphy TP, Passarelli J, Russmann S, Salamone SJ, Watson DE (2004) Pharmacokinetics and excretion of gamma-hydroxybutyrate (GHB) in healthy subjects. J Anal Toxicol 28(8):625–630
122. Couper FJ, Logan BK (2000) Determination of gamma-hydroxybutyrate (GHB) in biological specimens by gas chromatography–mass spectrometry. J Anal Toxicol 24(1):1–7
123. Lora-Tamayo C, Tena T, Rodríguez A, Sancho JR, Molina E (2003) Intoxication due to 1,4-butanediol. Forensic Sci Int 133(3):256–259
124. Elian AA (2000) A novel method for GHB detection in urine and its application in drug-facilitated sexual assaults. Forensic Sci Int 109(3):183–187
125. Mazarr-Proo S, Kerrigan S (2005) Distribution of GHB in tissues and fluids following a fatal overdose. J Anal Toxicol 29(5):398–400
126. Elian AA (2001) GC–MS determination of gamma-hydroxybutyric acid (GHB) in blood. Forensic Sci Int 122(1):43–47
127. Kaufmann E, Alt A (2007) Determination of GHB in urine and serum by LC/MS using a simple one-step derivative. Forensic Sci Int 168(2–3):133–137
128. Fung HL, Haas E, Raybon J, Xu J, Fung SM (2004) Liquid chromatographic-mass spectrometric determination of endogenous gamma-hydroxybutyrate concentrations in rat brain regions and plasma. J Chromatogr B Analyt Technol Biomed Life Sci 807(2): 287–291