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Original Article

Study of Gamma-Hydroxybutyric Acid (GHB) Concentrations in Postmortem Blood and Urine

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Abstract

Gamma hydroxybutyric acid (GHB) is present in blood and urine of the general population as an endogenous compound. The published concentrations in postmortem blood ranged from 0-168 mg/L in cases with no previous history of GHB use. Interpretation of GHB results should be carefully considered due to the wide distribution of endogenous concentrations.

The objectives of this study are to evaluate and verify the accuracy of a proposed published (50 mg/L) cut-off in 120 blood and 64 urine samples in postmortem cases selected randomly, and to identify GHB-related fatalities.

GHB was determned by gas chromatography–mass spectrometry (GC–MS) after extraction of the blood and urine in the presence of the internal standard GHB-D6.

The GHB concentration in majority of the blood samples (95%) was ≤ 50 mg/L, while in 81% it ranged from 10-50 mg/L. In 95% of the urine samples, the GHB concentration ranged from 10-20 mg/L while 82% of the samples had a concentration of < 10 mg/L. In cases where GHB intoxication was identified, GHB concentrations ranged from 264 to >500 mg/L.

The proposed published GHB concentration of 50 mg/L may be used as a cut-off to distinguish between natural endogenous concentrations and exogenous use, but this is not sufficient by itself. The detected GHB concentrations, both in vivo and in postmortem samples, require careful interpretation, not only due to its endogenous nature, but also due to the possibility of postmortem production and also due to its rapid metabolism and excretion.In order to distinguish the endogenous GHB concentration from those reflecting abusive GHB levels, defining a specific cut-off value in biological samples is very crucial. Other matrices, such as vitreous humour, femoral blood and hair must also be considered when interpreting postmortem GHB concentrations.

Key words: Forensic Science, GHB, GC-MS, Postmortem, Blood, Urine

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دراسة تراكيز حمض غاما هيدروكسي بوتريك (GHB) في عينات الدم والبول ما بعد الوفاة

المستخلص

يوجد حمض غاما هيدروكسي بوتريك (GHB) في الدم والبول عند الأشخاص العاديين كونه مركب داخلي يصطنعه الجسم، وإن

Introduction

Gamma hydroxybutyric acid (GHB) is a short chained fatty acid compound found in Mammalian tissues and in several brain areas [1]. It is present in blood and urine of the general population as an endogenous compound. GHB is a minor metabolite of γ-aminobutyric acid (GABA), which is an inhibitory neurotransmitter and is a central nervous system depressant. Figure-1 illustrates the metabolism of GHB along with the two main metabolic precursors of GHB, γ-butyrolactone (GBL) and 1, 4-butanediol (1,4BD). GHB is converted by GHB dehydrogenase to succinic semialdehyde then to succinic acid which becomes a substrate in the Krebs cycle and is metabolised to carbon dioxide and water [2]. It has been suggested that enzymatic conversion of succinic acid, GABA and putrescine are probably responsible for endogenous GHB production. In addition, glycolysis by bacteria may enhance endogenous GHB production. Bacteria can metabolize glucose to succinic acid via phosphoenolpyruvate and oxaloacetate. Succinic acid is converted to succinic semialdehyde that can be reduced to GHB by succinic semialdehyde reductase [3].

GHB has been used therapeutically as an anaesthetic and antidepressant agent. It causes relaxation and has the capacity to induce euphoria, short-term amnesia and sedation at high concentrations [3]. GHB is a medicinal product by definition of Article 1 of EC Directive 2001/83/EC5 and in the UK, GHB is controlled by the Medicines Act 1968 and associated regulations. The British National Formulary (BNF) lists Xyrem® as a hypnotic for use in treating narcolepsy (a rare sleep disorder characterised by excessive daytime sleepiness) with cataplexy (under specialist supervision). GHB was licensed in Europe in 2005 and in Canada and Switzerland for the same purpose. In 2002, the US FDA gave approval for Xyrem® (with GHB as an active ingredient) to be used in the treatment of cataplexy attacks in patients with narcolepsy. In only five EU Member States GHB is a licensed medicine for human use; GHB is used in France and Germany as a surgical anaesthetic and in Austria and Italy to treat alcohol withdrawal symptoms, and it has also been tested to treat opiate addiction. It is available in the Netherlands for this purpose. GHB has also been suggested for the treatment of fibromyalgia [4].

It has been highlighted that significant caution is needed when ingesting GHB/GBL, particularly in combination with alcohol, benzodiazepines, opiates, stimulants, and

التراكيز التي وجدت في عينات دم ما بعد الوفاة وفقاً للمراجع المنشورة تتراوح بين 0-160 mg/L، وذلك في الحالات التي لم تقترن بأي استخدام أو تعاطي ل (GHB). وإن تفسير تراكيز هذه المادة يجب أن يتم بحذر وبدقة نظراً للتوزع الواسع لنفس المادة ذات المصدر الداخلي من الجسم.

تهدف هذه الدراسة إلى تقييم الحد القطعي cut-off لمادة (GHB) والمقدر ب 50 mg/L والتحقق منه وذلك بدراسة 120 عينة دم ما بعد الوفاة، و 64 عينة بول ما بعد الوفاة، والتي تم اختيارها عشوائياً للكشف عن حالات الوفيات المرتبطة بـ (GHB).

لقد تمت الدراسة باستخدام جهاز الكروماتوغرافيا الغازية المقترنة بمطياف الكتلة (GC-MS)، بعد أن تم استخلاص عينات الدم والبول بوجود (GHB-d6) كعياري داخلي. ولقد أشارت النتائج بأن غالبية تراكيز (GHB) في عينات الدم ما بعد الوفاة المفحوصة (95%) كانت أقل من أو تساوي 50 mg/L، منها ما يعادل (81%) تتراوح فيها التراكيز بين 10-50 mg/L، أما بالنسبة لعينات البول ما بعد الوفاة المفحوصة فإن التراكيز تراوحت بين 10-20 mg/L في (95%) من العينات، منها (82%) كانت التراكيز فيها أقل من 10 mg/L. وفي الحالات التي سجلت كتسمم ب (GHB) تراوحت التراكيز بين 264 mg/L إلى ما يزيد عن 500 mg/L.

ومن خلال مناقشة النتائج المتحصّل عليها نجد أنه يمكن استخدام قيمة الحد القطعي المنشورة والمقدرة ب 50 mg/L وذلك للتفريق بين تراكيز المادة الطبيعية المنتجة داخل الجسم (endog-enous) وبين المادة التي تؤخذ عن طريق خارج الجسم (exoge-nous)، ولكن هذا ليس كافياً بحد ذاته. إن تراكيز (GHB) التي يتم كشفها في العينات السريرية أو عينات ما بعد الوفاة تحتاج إلى التفسير بشكل دقيق وحذر، وهذا ليس بسبب أن مصدر هذه المادة قد يكون داخلي ولكن أيضاً بسبب احتمالية تشكلها بعد الوفاة، وأيضاً بسبب أيضها وإطراحها السريعين. وهذا يؤكد أن تقدير الحد القطعي في العينات الحيوية يعتبر ذو أهمية بالغة بهدف التفريق بين تعاطي (GHB) ذي المصدر الخارجي و (GHB) ذي المصدر الداخلي. وبالنسبة للعينات الأخرى مثل الخلط الزجاجي والدم الفخذي والشعر فيجب أخذها بعين الاعتبار عند تفسير نتائج (GHB) في حالات ما بعد الوفاة.

الكلمات المفتاحية: الأدلة الجنائية، GHB, GC-MS، حالات ما بعد الوفاة، الدم، البول

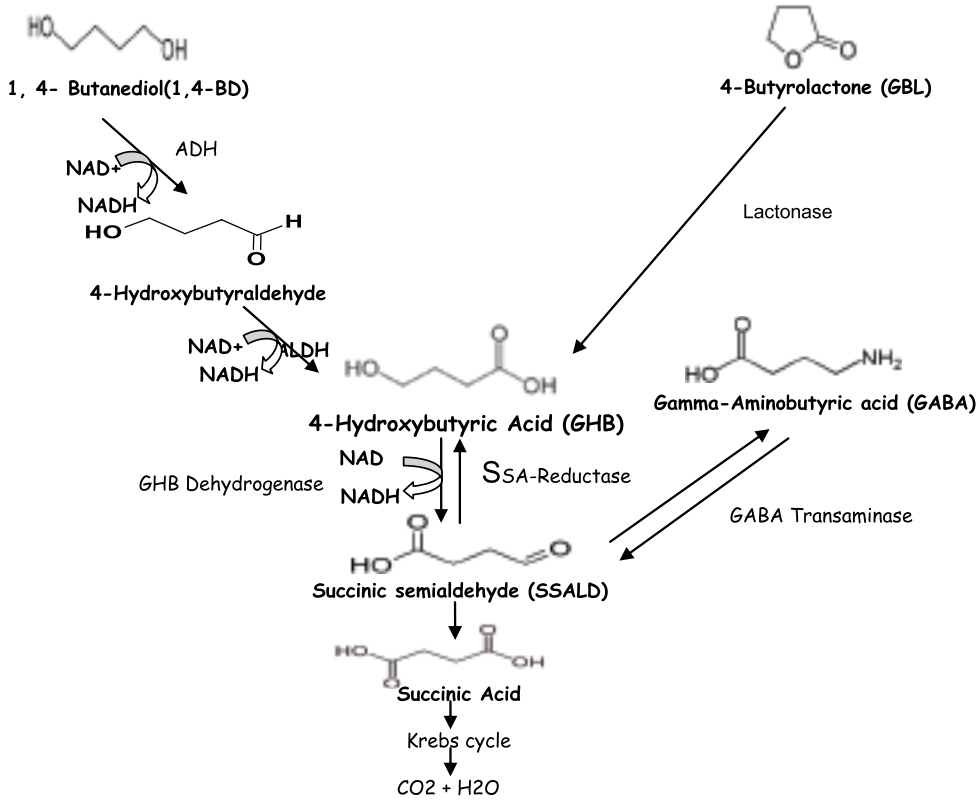

Figure 1- *In vivo metabolism of GHB, GABA, GBL and 1,4BD.*
ADH (Alcohol Dehydrogenase), ALDH (Aldehyde dehydrogenase), SSA-Reductase (Succinic semialdehyde reductase), SSA-Dehydrogenase (Succinic semialdehyde dehydrogenase), This Figure has been redrawn from Marinetti,JL [3].

ketamine due to their severe and unpredictable effects, including their addiction potential and withdrawal issues. The GHB analogue GHV (gamma-hydroxyvaleric acid) may also be seen as an alternative molecule to take, being advertised as a dietary supplement [4].

Individuals commonly associated with abuse of GHB include body builders who believe that GHB stimulates the release of growth hormones, club-goers for its sedation, muscle relaxation and other effects, drivers for recreational abuse and victims of drug-facilitated sexual assault [5]. GHB is often referred to in the media as the "date-rape drug" and is also known as "liquid ecstasy", however, it is not in the same drug class as methylenedioxymethamphetamine (MDMA), commonly referred to as "ecstasy" but simply refers to the similar effects experienced by users [3].

A growing number of overdose cases and/or sexual assaults with suspicion of GHB use have lead to an increased demand for toxicological analysis, and determination of GHB concentrations in biological samples for forensic purposes and becoming part of routine analysis in many forensic toxicology laboratories.

Several aspects need to be considered in relation to GHB concentrations in forensic toxicology and result interpretation, such as its postmortem behaviour in biological samples; endogenous production values, whether in vivo and in postmortem samples, sampling and storage conditions (including stability tests), the site from where the sample was taken because this may result in variable GHB concentrations due to, postmortem blood redistribution, longer storage time can also lead to higher sample concentrations, preservatives and additives may have an effect, storage temperatures and drugs such as valproate, phenobarbital, barbital and chlorpromazine may cause interactions with metabolic pathways [4-6].

Detection of exogenous GHB in body fluids requires collection of samples without delay. The timing is critical as traces of the drug disappear very quickly from the body with a detection time in blood of approximately 8 hours after ingestion and 12 hours in urine. In some cases, the recommended collection of blood and urine is no more than 48 hours after the offence is committed [7].

In postmortem blood samples, GHB is frequently

detected at levels that range from physiological to pharmacological concentrations, even in cases when GHB use is not suspected. The published postmortem GHB concentration in blood ranges from 3.2-168 mg/L in cases with no previous history of GHB use [1].

GHB has been found in postmortem biological fluids in therapeutic concentrations even when there was no history of prior consumption. Even though such findings have shown blood concentrations up to 200 mg/L, this data may have been influenced by storage conditions and by the specificity of the analytical technique used. Some studies have shown that the influence of the presence or absence of preservatives, as well as different storage temperatures, can be the reason for GHB concentrations increasing over time. GHB postmortem production is not limited to blood samples, but can also be observed in other biological fluids [6]. In one study, the exogenous increase of GHB concentration in postmortem blood was only temporary, reaching the maximum with a subsequent drop to immeasurable values. Another study reported that 43 mg/L was the highest GHB concentration in postmortem blood [8].

Concentrations in postmortem blood samples can reach significant values, even if the individual is a non-consumer; however, this production can be minimized by the use of sodium fluoride as a preservative in whole blood samples (1-5%), and with sample storage at -20°C.

Zvosec et al [9] published a case series of 226 GHB-related deaths with postmortem GHB concentrations ranging from 18 to 4,400 mg/L (mean 554 mg/L, median 347 mg/L, n= 64), demonstrating considerable overlap with reported concentrations from apparent endogenous GHB.

Busardo et al [1] reported that at postmortem, there is a strong correlation between post mortem interval (PMI) and GHB concentration in blood and urine. The results obtained in blood and urine samples showed a statistically significant difference ($p < 0.001$) in groups which were categorized according to PMI in the first analysis performed immediately after autopsy. They also found that there is no significant increase in GHB concentration when samples are stored at different temperatures for a period of one month; however, the lowest increase was at -20°C, so they recommended that freezing samples at -20°C is the ideal storage temperature for samples submitted for GHB analysis.

A positive correlation was shown in a study between postmortem time increasing (gap between death and sample collection) and GHB concentration in the same samples. But conversely, a proportional relationship between GHB concentration and extent of putrefaction was not observed [10]. GHB concentrations may increase after death in cases of advanced decomposition and when blood is collected from the heart. The GHB concentration due to postmortem formation was reported to reach 409 mg/L. High GHB concentrations were also reported in case samples where there was no evidence of GHB and/or GBL exposure [5].

A review of cases associated with GHB/GBL and 1,4-butanediol (1,4-BD) use, extracted from the UK's National Programme on Substance Abuse Deaths database between 1995 and September 2013, identified 159 cases of GHB/GBL associated fatalities. Postmortem blood concentration ranged from 0-6500 mg/L with a mean of 482 mg/L [4].

An elevation of the urinary concentrations of GHB has been reported in the case of a genetic disorder called GHB aciduria, due to a deficiency of succinic semialdehyde dehydrogenase. This leads to an accumulation of GHB in the urine. The reported concentration of GHB in this type of disorder was approximately or greater than 200 mg/L [2, 11-13]. Table-1 summarises the published GHB concentrations measured in different case types.

Regarding stability of GHB in biological samples, Stephens et al [21] observed a relatively minimal formation of GHB in blood samples stored in a refrigerator without sodium fluoride. In a recent study [8], the results show that concentrations of GHB in whole blood are stable during storage at 4°C for up to 6 months. Another study in plasma GHB found it to be stable at -20°C for up to 9 months, at room temperature for 48 hours, and after 3 freeze/thaw cycles. The processed samples stored at room temperature were found to be stable for 5 days and for 15 days at -20°C [9].

Berankova et al reported GHB concentrations up to 100 mg/L in postmortem blood [22]. It has been reported that the GHB detected in postmortem blood could be due to production during the interval between death and autopsy, rather than during storage at 4°C [6].

It has also been suggested [8] that there may be a correlation between GHB concentrations in whole blood and the corresponding postmortem intervals, whereas no correlation between GHB concentrations and storage periods was observed, if stored at -20°C. During 10 days, the GHB concentration had increased by 1.51 ± 1.15 μg/mL in 14 postmortem samples stored at 4°C.

In another study [22], the GHB stability of postmortem

Table 1- *GHB concentrations obtained from previous studies in antemortem and postmortem cases*

Reference	Matrix	Population/Status	GHB (mg/L)	N
Endogenous GHB ante-mortem and postmortem with no history of GHB/GBL use				
[14]	Urine	Antemortem urine	0.34-5.75	670
[11]	Urine	Patients with GHB aciduria	200	6
[15]	Blood	Postmortem	0-168	20
[5]	Blood (Cardiac)	Postmortem	0.4-409	71
	Blood (Femoral)	Postmortem	17-44	5
[16]	Blood	Postmortem	Mean was 4.6	23
[17]	Blood	Postmortem (Unpreserved)	0-158	26
	Blood	Postmortem (preserved)	0-193	125
Exogenous GHB (For cases where consumption of GHB/GBL was known)				
[18]	Urine	Volunteers after 10-hours from administration of 1 gram GHB	4	1
[19]	Blood	People arrested for driving under the influence of GHB	Mean=89, highest GHB conc. was 340	548
Fatal GHB concentrations with suspected GHB/GBL intoxication				
[20]	Blood	Postmortem	In excess of 300	49
[4]		postmortem	0-6500	159
This Study	Blood	Postmortem	Range from 264 to >500	5
	Urine	Postmortem	>500	2

urine and whole blood samples, in subjects with no previous GHB consumption history, using samples kept at 4°C, with sodium fluoride (NaF), was investigated at months 2 and 4. During the first two months, GHB concentrations increased by up to 30 mg/L, followed by a decrease the following two months to 6.5 mg/L. This study also in indicated that in vitro GHB production during storage is more substantial in postmortem samples than in ante-mortem materials. No concentrations of GHB above 3 mg/L were reported in antemortem serum specimens; whereas, in postmortem blood GHB concentrations, up to 100 mg/L were found. For longer storage times, lower temperatures and the use of preservatives (such as NaF) could decrease in vitro GHB formation in postmortem blood. Exogenous increase of GHB concentrations in postmortem blood was only temporary, reaching the maximum with a subsequent drop to immeasurable values [22].

It has been suggested that a comparison between paired preserved and unpreserved blood samples collected postmortem would provide an opportunity to investigate the role of a preservative in preventing or decreasing in vitro formation of GHB in postmortem blood [17].

There has been a lengthy debate about what should

be the appropriate minimum concentration for GHB and its analogues in human ante- and postmortem samples to confirm endogenous use. An evaluation of the proposed cut-off concentrations for different biological samples, such as whole blood, plasma, serum, urine, saliva, bile, vitreous humour and hair is important to differentiate exogenous from endogenous GHB [4, 6]. Table-2 summarizes the published cut-offs proposed by other researchers.

The recommended published cut-off concentrations of GHB in ante-mortem urine and blood were 10 mg/L and 50 mg/L respectively [14]. In postmortem femoral blood and urine for reporting positive results, the cut-off was proposed at 30 mg/L [20]. In another study of postmortem cardiac blood, the GHB cut-off concentration was reported at 50 mg/L [5]. The most common cut-off concentration for urinary GHB reported in several studies to differentiate endogenous from exogenous was 10 mg/L. This can be applied to the samples that were properly stored, and excludingthe GHB aciduria cases [2, 14, 23].

Materials and Method

The analytical method for the determination of GHB in blood and urine was modified and validated from a method previously validated and published and was initially used for the simultaneous analysis of beta-hydroxybutyrate (BHB) and GHB in blood and urine using deuterated GHB as the internal standard (GHB-D6). BHB is a ketone body used as a biomarker of alcoholic or diabetic ketoacidosis [26]. Validation of the method was in accordance with recommended best practice [27]. This method has a wide

Table 2-*Summary of the proposed GHB cut-offs published in the literature*

Reference	Matrix	Population/Status	GHB cut-off (mean) Range
[6]	Blood	Ante-mortem	(4) 1-5 mg/L
	Urine	Ante-mortem	(10) 3-10 mg/L
	Hair	Ante-mortem	1-14 ng/mg
	Blood (Femoral)	Postmortem	17-44 mg/L
	Urine	Postmortem	(20) mg/L
	Vitreous Humour	Postmortem	(84) 50-85 mg/L
[2, 14, 23]	Urine	Ante-mortem	10 mg/L
[14]	Blood	Ante-mortem	50 mg/L
[10, 16, 20]	Blood (Femoral)	Postmortem	30 mg/L
[24]	Blood	Postmortem	50 mg/L
	Urine	Postmortem	20 mg/L
	Vitreous Humour	Postmortem	7 mg/L
[25]	Blood (venous)	Postmortem	30 mg/L
	Urine	Postmortem	30 mg/L
	Cerebrospinal fluid (CSF)	Postmortem	30 mg/L
[5]	Blood (Cardiac)	Postmortem	50mg/L

linear range from 5-500 mg/L compared to other published methods, with the advantage of reducing reanalysis of samples with high GHB concentrations. In addition, the limit of detection is low and the extraction from blood using acetonitrile produces a cleaner extract.

Reagents and Standards

GHB-D6, the internal standard, was purchased from Cerilliant; 4-hydroxybutyrate (GHB) from Sigma; Millipore water, 2 ml polyethylene microcentrifuge vials, ethyl acetate of HPLC grade, N, and O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) from Fluka and Aldrich. Acetonitrile, methanol and ethyl-acetate were HPLC grade, sulphuric acid 98%. All solvents and acid were from (BDH) VWR International Ltd. Diluted sulphuric acid was prepared at a concentration of 0.025 M. A stock standard of GHB at a concentration of 2 mg/mL was prepared in water. Internal standard of GHB-D6 was prepared at a concentration of 10 mg/L in methanol.

Source and Preparation of Blank Blood and Urine

Blank drug-free blood used for preparing quality control materials was composed of expired packed red blood cells and was obtained from the blood bank at the Western Infirmary Hospital, Glasgow. Blank blood was diluted at a ratio of 1:1 with saline solution. Blank urine was obtained from a healthy adult volunteer with no history of alcohol consumption. GHB concentrations in the blank samples were compared to the limit of detection (LOD) and lower limit of quantification (LLOQ) in both matrices.

Quality control samples

Spiked blood and urine were prepared at concentrations of 50 and 300 mg/L and were then extracted with each batch. The coefficient of variance (CV) was calculated for each QC concentration with an acceptable CV < 10%. Batches were deemed acceptable when QC concentrations were within the mean ± 2 standard deviations (SD).

Instrumentation and Chromatography Conditions

Gas chromatography-mass spectrometry (GC-MS) was utilised with an Agilent GC-MS 7890A GC system and 5975 MSD, with a triple axis detector and ChemStation software (Agilent, UK). The column used was a DB-5+ DG capillary column (30m x 0.25 mm I.D., 0.25 μm film thickness) purchased from Agilent Technologies Ltd., UK. The initial oven temperature was 60ºC, held for two minutes, increased to 180ºC at 20ºC/min, and then further increased to 250ºC at 50 ºC/min and held for a further 1 minute. The total run time was 10.3 minutes. The source temperature was 200ºC; the transfer line temperature was 250ºC; the injector base temperature was 250ºC, using the split mode, and the carrier gas flow rate was 1.2 mL/min.

Data were collected in full-scan mode and the ions monitored were: m/z 233, 117, and 204 for GHB, and m/z 239, 241 for the internal standard GHB-D6. The bolded underlined ions were used for quantification.

Extraction Method

100 μL of blood was transferred to a 2mL snap-top polypropylene microcentrifuge tube, and 100 μL of internal standard (10mg/L) was added to all samples, in addition to 500 μL acetonitrile as the extraction solvent.

100 μL of urine was transferred to a 2mL snap-top polypropylene microcentrifuge tube, and 100 μL of internal standard (10mg/L) and 100 μL of 0.025M sulphuric acid were added to all samples, in addition to 1mL ethyl acetate as the extraction solvent.

All blood and urine samples were vortexed for 30 seconds and then centrifuged at 1500 rpm for 15 minutes. The solvent layer was collected and transferred to a clean vial and evaporated at 45ºC±1 to dryness with nitrogen. For the derivatization step, 75 μL of BSFTA +1% TCMS was added to all samples, mixed and heated at 90ºC for 10 minutes. Samples were transferred to GC vials and 2 μL injected on column.

Case Samples

Postmortem femoral blood (N=120) and urine (N =64) samples were selected randomly over a period of one month from the cases submitted to the Toxicology Laboratory within Forensic Medicine and Science at the University of Glasgow. All blood samples were preserved (0.2% sodium fluoride) and stored at 4ºC ±1 prior to analysis. Basic demographic information was also summarised including gender, age and the cause of death. This information was not always available for external postmortem cases carried out by a forensic pathologist who was not based within Forensic Medicine and Science.

Statistical calculations of mean, median and standard

deviation (SD) were calculated for all samples with no history of GHB/GBL-related death. All results were evaluated in comparison with published endogenous GHB concentrations. Case samples with high GHB concentrations were also identified. Causes of death were reviewed, with all case samples analysed.

Method Validation

Linearity

The linearity was achieved by spiking nine blank blood and urine samples with GHB at concentrations ranging from LLOQ - 500 mg/L. The linear measuring range was generated by plotting the peak area ratio of the GHB to the GHB-D6 of each point versus the concentration. The correlation of coefficient (R2) should be greater than 0.99 (Fig 2).

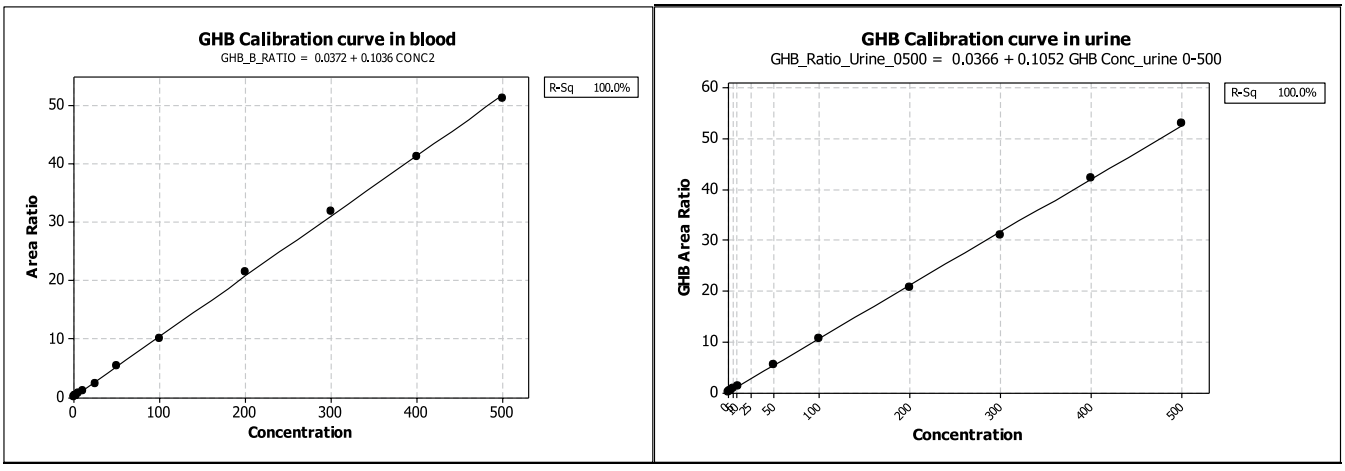

Figure 2- *The GHB calibration curve in blood and urine*

concentration on the standard curve of the analyte that can be accurately and precisely measured. LLOQ values are calculated using the same method of LOD but using 10 times the standard error of the regression line.

LOQ was calculated using the following equations:

YLOQ=YB+10SB
LOQ= (YLOQ-YB)/m

Where YLOQ is the standard error, YB is the intercept, and m is the gradient.

Method Precision

Intra-day Precision (Within Day Precision)

Six spiked controls were prepared at two different concentrations (50 and 300 mg/L) and were extracted and injected on the same day. The CV was calculated and the acceptable value was < 10%.

Inter-day Precision (Between Day Precision)

Ten blood and urine points of control samples were prepared at two different concentrations (50 and 300 mg/L) and were then extracted and injected on different days. The CV was calculated and the acceptable value was <10%.

Accuracy, Recovery and Efficiency

The accuracy of the method was determined by calculating the average percentage of six measured values of the extracted spiked control samples to the theoretical value at two concentrations (50 and 300 mg/L) in blood and urine.

Accuracy (%) = (Peak area ratio of extracted samples

Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

The LOD is the lowest concentration of the analyte that produces a response detectable above the background noise. LOD can be assessed by analysing spiked samples with decreasing concentrations of GHB within the expected range of the LODs. Regression analysis was undertaken, and the LOD and LLOQ were calculated in accordance with Miller and Miller [28].

LOD was calculated by using the following equations:

YLOD=YB+3SB
LOD = (YLOD-YB)/m

Where YLOD is the standard error, YB is the intercept, and m is the gradient.The LLOQ is defined as the lowest

(Internal standard added in the beginning of the extraction/ amount of the theoretical or nominal amount) x100

The recovery was assessed by spiking blood and urine in triplicate at two concentrations of 50 and 300 mg/L. Each concentration was extracted three times without the internal standard present. The internal standard was added to all samples before evaporation. The recovery was determined by comparing the percentage of peak area ratio of extracted standard to the peak area ratio of the unextracted standard.

Recovery (%) = (Peak area ratio of extracted samples (Internal standard added before evaporation) / peak area ratio of unextracted standard) x100

The efficiency of the method was calculated at two concentrations of 50 and 300mg/L. Each concentration was extracted in triplicate with presence of the internal standard. The efficiency was determined by calculating the percentage of the extracted spiked standards with presence of the internal standard to the peak area ratio of the unextracted standard. [29]

Efficiency (%) = (Peak area ratio of extracted samples, internal standard added in the beginning of the extraction) / (peak area ratio of unextracted standard) x100

Results

Method Validation Results

Linearity: GHB was found to have a linear response over the concentration range of LLOQ-500 mg/L in both blood and urine. The correlation of coefficient R2 was greater than 0.99.

LOD and LLOQ Results: The LOD of GHB in urine and blood was 1mg/L and the LLOQ was 2 and 4 mg/L respectively. Due to the endogenous nature of GHB, results less than 10 mg/L were reported as negative. Concentrations above the upper limit of quantification, 500 mg/L, were reported as >500 mg/L.

Figure-3 indicates the GHB chromatogram of blank samples overlaid with spiked blood and urine at low concentrations (2, 5 and 10 mg/L) to show that there was no interference from the matrix blank due to the endogenous nature of GHB.

Method Precision

Intra-day and Inter-day Precision

Table -3 shows The CV of intra-day precision and inter-day precision. All CV were <10%.

Table 3- *GHB intra-day and inter-day precision in blood and urine*

	Intra-day precision CV% (n=6)		Inter-day precision CV% (n=10)	
Matrix	50 mg/L	300 mg/L	50 mg/L	300 mg/L
Urine	1.7	1.5	2.9	2.7
Blood	2.3	3.1	3.3	5.6

Recovery, Efficiency and Accuracy Results

Table-4 summarises the recovery, efficiency and accuracy results. Recovery of GHB ranged between 40-50% in blood and urine. The efficiency of the method was between 83 and 99% and the accuracy was between 89 and 104%.

Quality Control Results

Table-5 summarises the CV of different QC concentrations and were all acceptable < 10%. Quality control ranges were also calculated. Figure-4 illustrates the GHB quality control charts at 50mg/L and 300mg/L in blood and urine.

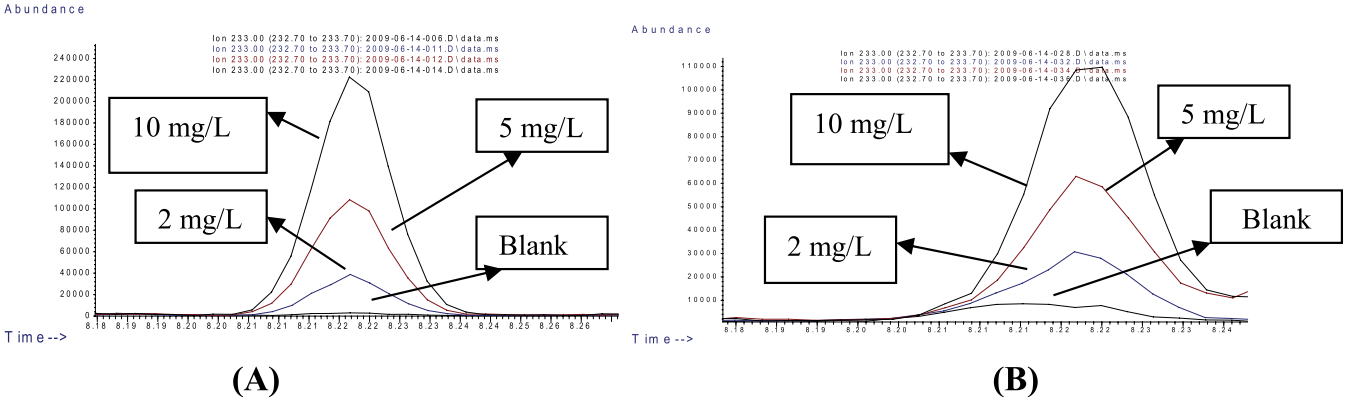

Figure 3- *GHB extracted ion chromatograms of blank (A) blood and (B) urine overlaid with blood and urine spiked at concentrations of 2, 5 and 10 mg/L.*

Case samples results

Figure-5 illustrates a typical chromatogram and spectra for GHB in postmortem case samples and the internal standard GHB- D6 extracted from blood. Table-6 summarises the postmortem cases for all GHB concentrations in urine > 10 mg/L and all blood cases with GHB concentration > 50mg/L not including those with histories of GHB/GBL intoxication.

The ranges of blood and urine GHB concentrations in blood and urine in case samples, not including those with histories of GHB intoxication, were (0 - 82 mg/L) and (0-38 mg/L) respectively. Mean and median for blood and urine were (20,19 mg/L) and (5,0 mg/L) respectively. Figure-6 illustrates the frequency of GHB concentrations (mg/L) in postmortem blood and urine in cases with no history of GHB and/or GBL Misuse. In 95% of the urine samples, the GHB consemtration ranged from 10-20 mg/L, 82% had <10 mg/L, and 5% ranged from 21-50 mg/L. The majority of blood samples (95%) had a GHB concentrations of ≤ 50 mg/L, 81% ranged from 10-50 mg/L, 13% were at a concentration of <10 mg/L and about 5 % ranged from 51-82 mg/L

Table-7 shows the high GHB concentrations in postmortem case samples with a history of suspected GHB intoxication. The range of GHB concentrations in blood and urine were 264 to >500 mg/L, in urine there were only two samples and they were both > 500 mg/L.

Discussion

Method Validation Discussion

The recovery of GHB was low and ranged between 40 and 50% in blood and urine. However, the efficiency of the method was between 83 and 99%, and the accuracy was between 89 and 104%. The efficiency and accuracy have been also been indicated from the QC results which were within acceptable CV limits.

Case Sample Results Discussion

Significant variations in the GHB concentrations in postmortem blood have been reported in the literature, even in cases when GHB use is not suspected. The broad range of GHB concentrations in postmortem blood could be due to formation of GHB between death and collection of samples due to enzymatic or bacterial actions as reported earlier in the introduction of this study.

In 95% of case samples, postmortem blood (n=106) and urine (n=58) GHB concentrations were < 50 mg/L and < 20 mg/L respectively. Only 5 % of blood samples had GHB concentrations in excess of 50 mg/L and less than 100 mg/L and 5% of urine samples had GHB concentrations in excess of 20mg/L and not exceeding 50 mg/L. The proposed cut-off of 50 mg/L for blood and

Table 4- *Recovery, efficiency and accuracy of GHB*

	Recovery (%)		Efficiency (%)		Accuracy (%)	
Matrix	50 mg/L	300 mg/L	50 mg/L	300 mg/L	50 mg/L	300 mg/L
Urine	40	41	99	96	95	89
Blood	47	50	86	83	95	104

Table 5- *Quality Control ranges of GHB*

	QC Ranges			
	Blood		Urine	
	50 mg/L	300 mg/L	50 mg/L	300 mg/L
Mean	49	293	50	314
Standard Deviation (SD)	2.61	20.11	3.12	22.42
CV%	5.4	6.7	6.3	7.1
Mean ± 2SD	43-54	253-333	44-56	270-359

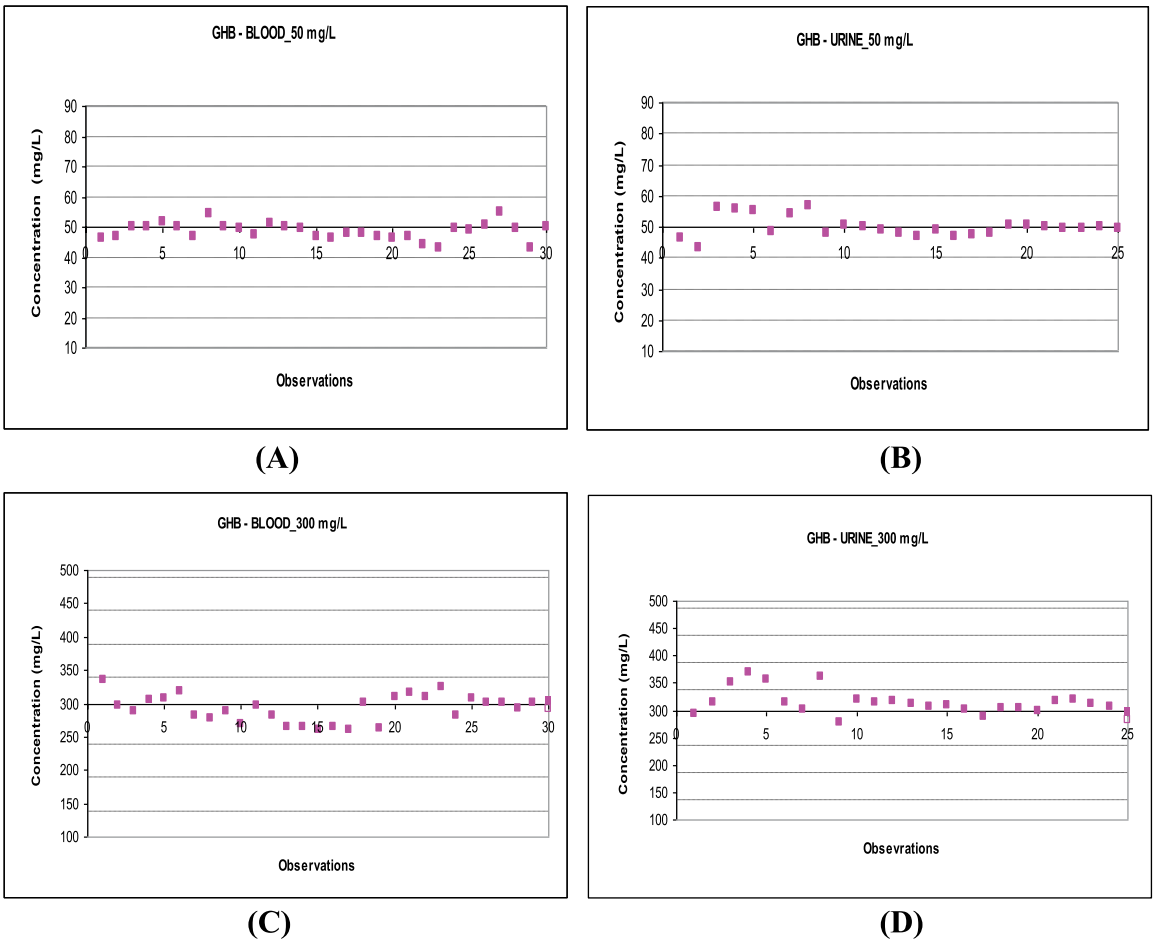

Figure 4- *GHB quality control charts at 50mg/L (A and B), 300mg/L (C and D) in blood and urine, respectively*

20 mg/L for urine to differentiate between exogenous use and endogenous formation of GHB effectively identified endogenous concentrations in the vast majority of cases. A recent study reported by Korb and Cooper [24] included a large number of postmortem cases (n=387) submitted to the toxicology laboratory specifically requesting the analysis of the ketoacidosis biomarker, beta-hydroxybutyrate (BHB). No reference to GHB use was identified in any of the case files. The cut-off in this study was in agreement with this published study [17], where the majority of samples 90.7 % (n=351) were ≤ 50mg/L with mean and median concentrations of 28 and 24 mg/L respectively, and 9.3% of cases were in excess of 50 mg/L. The median GHB concentration in blood calculated in this study was (19 mg/L), and it is approxiamtely 26 % less than that reported in Korb and Cooper's study [24]. However, the majority of postmortem blood samples in both studies were ≤ 50 mg/L and they are comparable with previous published studies. Analysis of GHB in postmortem urine in addition to blood samples may give supporting information when interpreting GHB because urine is expected to have lower GHB concentrations due to less postmortem generation.

Several studies have been undertaken to obtain reliable data for endogenous urinary GHB concentrations in humans. The endogenous urinary GHB concentration from a previous published study ranging of 0–6.63 mg/L [30, 31]. Another study of 670 ante-mortem urine samples reported GHB concentration ranged between 0.34 and 5.75 mg/L (mean: 3.08 mg/L; median: 3.00 mg/L) [14]. Another study reported an endogenous GHB concentration range of 0–2.74 mg/L [2]. Shima, et. al, 2005 [23], reported that the endogenous GHB concentrations in healthy volunteers' urine was, 0.10–2.68 mg/mL, while in diabetic patients GHB ranged from 0.14–124 mg/mL. It has also been indicated that a certain degree of in vitro GHB production in urine occurs, though this is less than that seen with the blood. It is also

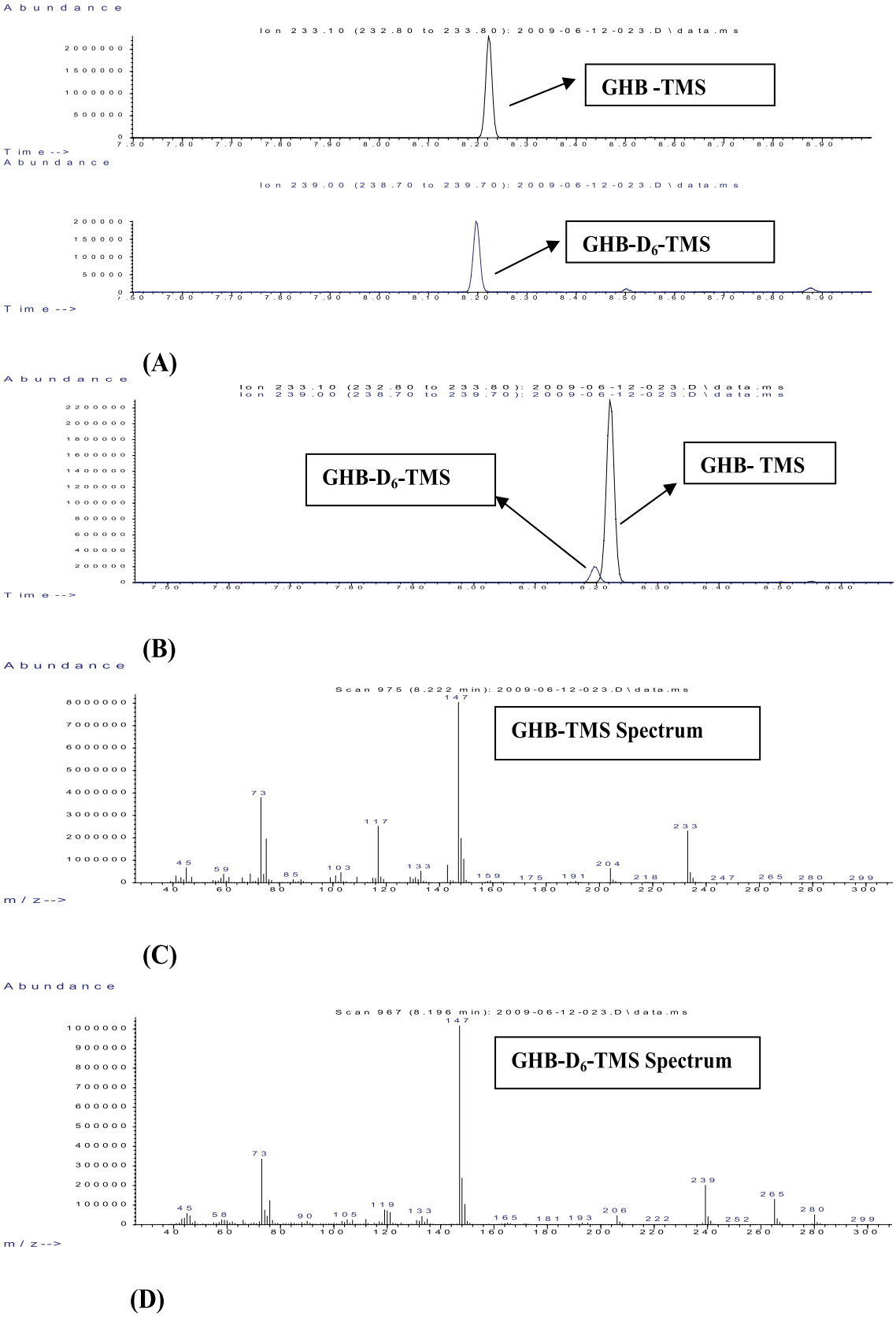

Figure 5 - *(A), (B) and (C), (D) are the selected ion chromatograms of GHB-TMS, GHB-D6-TMS and their spectra in a postmortem blood case sample containing GHB concentrations >300 mg/L.*

Table 6- *Postmortem cases with GHB concentration >10 mg/L in urine and >50 mg/L for blood, excluding cases with suspected GHB/GBL intoxication.*

Case #	Gender	Age	Urine GHB Results ≥ 10 mg/L	Cause of Death
6	F	78	14	Inhalation of smoke and fire gases in a house fire and hypertensive and valvular heart disease
9	F	58	12	Suspected alcohol related death
26	M	81	23	Acute pylonephritis Coronary artery atheroma
54	M	63	15	unacertained (Pending further investigation)
62	F	41	19	Methadone and dehydrocodone intoxication Coronary artery atheroma
64	M	61	18	Hanging
72	M	44	21	Fatty degeneration of the liver Chronic alcoholic abuse
99	M	38	38	Undetermined
100	M	80	18	Hypothermia
104	M	61	22	Ischaemic heart disease and gastrointestinal haemorrhage from erosive oesophagitis, Fatty degeneration of the liver due to chronic alcohol abuse
Case #	**Gender**	**Age (years)**	**Blood GHB Results ≥ 50 mg/L**	**Cause of Death**
78	n/a	n/a	50	External case, unascertained
101	M	25	54	Unascertained
102	M	36	65	Unascertained
109	M	39	56	Methadone and alcohol Intoxication
112	M	42	82	Ischaemic heart disease, Coronary artery atheroma Insulin dependent diabetes mellitus
120	M	n/a	59	Fatty degeneration of the liver, Chronic alcohol abuse

reported that the difference or variation of urinary GHB concentrations between published studies could be due to differences in the nature of subject population, eating and drinking tendencies, or in vitro GHB production before analysis.

In this study, the majority of the urine results (95%) ranged from 10-20 mg/L. Only 5% ranged between 21-50 mg/L with no urine results in excess of 50 mg/L. A cut-off of 10 mg/L is appropriate when the specimen is stored in the refrigerator. GHB aciduria and diabetic case samples should be considered and excluded to quantify the basal concentration of GHB [23].

The suggested cut-offs reported in the literature (>50, 30 and 10 mg/L) are not to be seen as rigid requirements, but as aids to interpretation. Kintz et al [5] argued that detection of GHB in urine is not necessarily indicative of use, and that concentrations in blood above 50 mg/L alone are insufficient to prove use.

Some authors recommend analysis of urine in addition to blood samples because it is expected to have lower GHB concentrations due to less postmortem generation. Other authors suggest vitreous humor as the specimen of choice, in addition to femoral vein blood, but there is limited comparative data for this fluid. It has been reported that some psychoactive substances might have an influence on the concentrations of GHB found in fatalities.

The concentrations of GHB in postmortem blood and urine that were associated with a background and cause of death of GHB/GBL intoxication had high or fatal concentrations ranging from 264 mg/L to a value >500 mg/L. The high value of GHB was also indicated in case samples where there was no evidence of GHB and/or GBL exposure. In non-fatal intoxications, concentrations as high as 551 mg/L have been reported whilst fatalities with concentrations of 303 mg/L have been recorded [32].

The potential role of decomposition was difficult

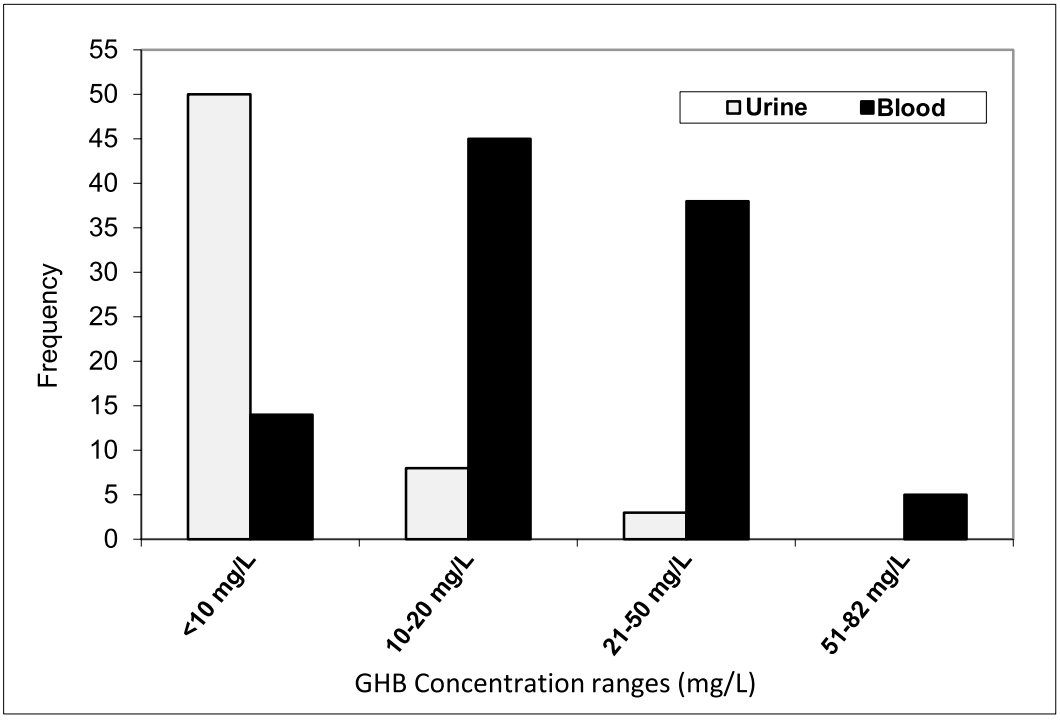

Figure 6- *Frequencies of GHB concentrations (mg/L) in postmortem blood and urine in cases with no history of GHB and/or GBL abuse*

to investigate due to a lack of standardization as to how decomposition changes were reported. In addition, it is not possible to assess the effect PMI may have had on the GHB concentrations due to the unreliability of the available data [25].

In postmortem peripheral blood samples, high concentrations of GHB could be measured, even in cases without GHB ingestion. These concentrations overlap with the range of reported fatal GHB intoxications with concentrations of 27 to 2937 mg/L and could possibly lead to misinterpretation as intoxication [33].

This made the interpretation more difficult to distinguish between the endogenous and exogenous concentrations of GHB. The postmortem formation of GHB, its quick elimination from the body, lack of the data concerning PMI, improper storage of samples and the effect of preservatives all contribute to the challenge of interpreting GHB concentrations in postmortem cases. Interpretation of the majority of cases with elevated GHB will clearly indicate endogenous formation of GHB, but there are always cases that will continue to present a challenge [6, 17].

Conclusion

The proposed cut-offs of 50 mg/L for blood and 20 mg/L for urine to differentiate between exogenous use and endogenous formation of GHB were effective in the identification of endogenous concentrations in the vast majority of cases in this study but not in all. Other matrices, such as vitreous humour, femoral blood and hair

Table 7- *High GHB concentrations in postmortem samples.*

Case #	Gender	Age	GHB (mg/L)		Background
			Blood	Urine	Cause of Death
36	M	34	>500	>500	Gamma-hydroxy butyrate (GHB) intoxication
103	n/a	n/a	369	n/a	Suspected GHB/GBL intoxication
106	n/a	n/a	300	n/a	Suspected GHB/GBL intoxication
108	n/a	n/a	>500	>500	Suspected GHB/GBL intoxication
118	M	n/a	264	n/a	Suspected GHB/GBL intoxication

must also be considered, as recommended by Kintz et al [4] when interpreting postmortem GHB concentrations. Knowledge of the time interval, the time of sample collection and the extent of decomposition may also provide supportive information for result interpretation.

The concentrations of GHB in post mortem blood and urine that were associated with a background and cause of death of GHB/GBL intoxication had higher or fatal concentrations ranging from 264 to a value >500 mg/L.

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