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Simultaneous analysis of gamma-hydroxybutyric acid and its precursors in urine using liquid chromatography–tandem mass spectrometry

Michelle Wood[a,*], Marleen Laloup[b], Nele Samyn[b], Michael R. Morris[a], Ernst A. de Bruijn[c], Robert A. Maes[c], Michael S. Young[d], Viviane Maes[e], Gert De Boeck[b]

[a] *Waters Corporation, MS Technologies Centre, Micromass UK Ltd., Atlas Park, Simonsway, Wythenshawe, Manchester M22 5PP, UK*
[b] *National Institute of Criminalistics and Criminology (N.I.C.C.), Section Toxicology, Vilvoordsesteenweg 98, 1120 Brussels, Belgium*
[c] *Department of Human Toxicology, Utrecht Institute of Pharmaceutical Sciences (UIPS), University of Utrecht, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*
[d] *Waters Corporation, 34 Maple St., Milford, MA, USA*
[e] *Department of Clinical Chemistry-Toxicology, Academic Hospital, Free University of Brussels, Brussels, Belgium*

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Abstract

We have developed a rapid method that enables the simultaneous analysis of gamma-hydroxybutyrate (GHB) and its precursors, i.e. gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) in urine. The method comprised a simple dilution of the urine sample, followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Chromatographic separation was achieved using an Atlantis dC_{18} column, eluted with a mixture of formic acid and methanol. The method was linear from 1–80 mg/L for GHB and 1,4-BD and from 1–50 mg/L for GBL. The limit of quantification was 1 mg/L for all analytes. The procedure, which has a total analysis time (including sample preparation) of less than 12 min, was fully validated and applied to the analysis of 182 authentic urine samples; the results were correlated with a previously published GC–MS procedure and revealed a low prevalence of GHB-positive samples. Since no commercial immunoassay is available for the routine screening of GHB, this simple and rapid method should prove useful to meet the current increased demand for the measurement of GHB and its precursors.
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1. Introduction

Gamma-hydroxybutyrate (GHB) is an endogenous metabolite of gamma-aminobutyric acid (GABA). Since the 1960s GHB has been used clinically as an intravenous anaesthetic and as a treatment for narcolepsy, alcoholism and opiate withdrawal. Over the last few years, GHB has been gaining popularity amongst club-goers as a recreational drug (liquid ecstasy), where it is taken for its ability to produce feelings of euphoria and to enhance sexuality [1–3]. As a result of its potent prosexual effects, GHB has also been increasingly implicated in drug-facilitated sexual assault (DFSA) [4,5]. The ingestion of the chemical precursors of GHB, i.e. gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) also results in similar physiological effects since they are rapidly converted to GHB in the body [6]. GHB itself is rapidly metabolised and has a half-life of 20–35 min, depending on the dose [6–8]. The concentrations that result from a typical dose of GHB can be cleared from the blood of living subjects within 6 h of administration [9]. In urine, the concentrations of GHB tend to parallel those found in the blood but are ∼10-fold higher [10]. Thus, the window of GHB detection can be extended to ∼12 h when urine is used as the physiological sample [8]. Consequently, urine is the recommended biological specimen especially in cases of suspected DFSA [11].

Positive GHB findings must be interpreted with caution since it is a component of normal mammalian metabolism and endogenous levels are found in all biological tissues. Indeed, 'normal' urinary concentrations have been shown to vary considerably [12–14], such intra- and inter-individual

* Corresponding author. Tel.: +44 161 435 4100; fax: +44 161 435 4444.
E-mail address: michelle_wood@waters.com (M. Wood).

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variation may be the result of biochemical, drug-induced or dietary factors [14]. Furthermore, studies have also shown that the storage conditions used for urine samples collected from living persons, can influence the amount of in vitro production of GHB [15,16]. However, to date, none have demonstrated artifactual production of GHB to levels that might be consistent with those obtained following GHB ingestion. The consensus is that in the absence of exogenous GHB ingestion, urinary concentrations are below 10 mg/L; consequently, this level has been proposed as the interpretative cut-off concentration, based on several large-scale studies [5,14,16,17].

A variety of analytical methods have been described for the analysis of GHB in biological samples and seized drug preparations. Most of these are based on gas chromatography (GC) [16–20]. However, owing to their low molecular weight, high polarity and thermal instability, GHB and its precursors are not directly amenable to analysis by GC and therefore require derivatisation or modification prior to GC analysis. One such modification is the cyclization of GHB to GBL under acidic conditions [17,18]. However, whilst conversion to the lactone improves chromatography, it prevents the differentiation of GHB and GBL. In order to circumvent this limitation, LeBeau et al. [20] proposed a two-step procedure. Following an initial screen, i.e. using headspace GC-flame-ionisation-detection, positive samples were reanalysed by GC in conjunction with mass spectrometry (GC–MS). However, for the differentiation of GBL and GHB, *two* further analyses per sample were necessary. Consequently, this procedure is not really suitable for the quantification of large numbers of samples.

Another analytical approach involves the derivatisation of the GHB, e.g. using trimethylsilyl (TMS) prior to GC or GC–MS (/MS) analysis [9,16,21]. These techniques generally provide sufficient sensitivity to quantify down to endogenous levels of GHB. However, they also tend to be quite time-consuming and therefore, again, not ideally suited for the analysis of large numbers of samples. Furthermore, most of those reported do not allow the simultaneous analysis of GHB *and* the precursors.

Several investigators have used capillary zone electrophoresis (CZE) for the analysis of GHB [22,23]. Bortolotti et al. recently described a method that used minimal sample preparation, i.e. dilution only, prior to a short CZE-UV analysis. However, with a limit of detection of only ∼24 mg/L in urine, this technique does not allow for the simultaneous analysis of the precursors or the quantification of physiological GHB levels. Nevertheless, its simplicity may prove useful for the screening of large numbers of putative GHB-overdose cases [23].

Some methods based on liquid chromatography (LC) have also been described. These methods combined LC with UV [24] or single stage MS [25] detection and proved useful for the analysis of drug preparations. However, they are unlikely to be useful for the analysis of GHB in biological samples due to their limited sensitivity.

Raised awareness of the effects of these drugs and their potential for misuse, in addition to their ease of availability, has resulted in a dramatic increase in the demand for their analytical determination in both biological specimens and putative drug preparations [12]. To date however, no immunoassays are available for the rapid screening of biological samples for GHB and its precursors and GHB is not routinely found using systematic toxicological analysis. Therefore many cases involving GHB may be missed in hospitals and forensic institutes unless specifically requested. Clearly, there is a requirement for simple and rapid analytical procedures to meet the current increased demand for analysis. The purpose of this study was therefore to develop and validate a sensitive technique that would enable the simultaneous quantification of GHB, GBL and 1,4-BD in urine.

2. Experimental

2.1. Chemicals

Individual ampoules of GHB and GHB-d6 (both standards supplied as the sodium-salt at a concentration of 1 g/L in methanol) were purchased from LGC Promochem (Teddington, UK). GBL-d6 (1 g/L in acetonitrile) was from the same supplier. GBL and 1,4-BD (minimum purity 99% for both liquids), alpha- and beta-hydroxybutyric acid were from Sigma–Aldrich (Poole, UK).

All solvents were HPLC-grade and from Fluka (Gillingham, UK) or Fisher Scientific (Loughborough, UK).

2.2. Deuterated internal standard (IS) solution, calibrators and quality control (QC) samples

A mixed IS solution was prepared by adding GHB-d6 and GBL-d6 to deionised water to yield a final concentration of 2 mg/L. This solution was stored at 4 °C and used within 2 weeks of preparation.

Control urine, used for the preparation of calibrators and QC samples, was obtained from LGC Promochem. A mixed spiking solution was prepared by adding GHB, GBL and 1,4-BD to deionised water to yield a final concentration of 800 mg/L. This solution was further diluted (with deionised water) to give a range of mixed spiking solutions, i.e. 10, 20, 50, 100, 200 and 500 mg/L. These were subsequently used to spike control urine and to yield a series of calibrators enriched with GHB, GBL and 1,4-BD at the following concentrations; 0, 1, 2, 5, 10, 20, 50 and 80 mg/L.

Low and high QC samples were prepared by spiking control urine with mixed spiking solutions to give urinary concentrations of 4 and 40 mg/L, respectively.

2.3. Biosamples

One hundred and eighty-two authentic human urine samples from club-goers, attending a post dance-club ‘chill-out’

venue, were routinely screened for common illicit drugs by FPIA (Axsym, Abbott Diagnostics). Additional screening for GHB was demanded by the prosecutor. After routine analysis with GC–MS had been completed (and after the samples had been anonymized), LC–MS/MS analysis of GHB and its analogues was performed. An aliquot of 1 mL was stored at −20 °C until analysis. Urinary pH was estimated using pH test strips (Sigma–Aldrich).

2.4. Sample preparation for LC–MS/MS

2.4.1. Dilution

Urine samples (either calibrators, QCs or authentic samples) were diluted 1:20 with the deuterated IS solution and vortex-mixed for 10 s. Diluted samples were transferred to an autosampler vial for LC–MS/MS.

2.4.2. OASIS cartridge clean-up

Five hundred microlitres of deuterated IS solution were added to 250 μL of urine sample (either calibrator, QC or authentic sample). The sample was made up to 1 mL using water and vortex-mixed for 10 s before applying to a pre-conditioned OASIS MCX solid-phase extraction (SPE) cartridge (60 mg/3cc, Waters) for sample clean-up. The cartridge was pre-conditioned with 1 mL volume of methanol followed by 1 mL of water. The diluted urine sample was applied to the cartridge and the unbound fraction discarded in each case. Five hundred microlitres of 0.1% formic acid was applied to each cartridge, the eluate collected and then transferred to an autosampler vial for LC–MS/MS analysis.

2.5. LC–MS/MS

2.5.1. Chromatography

LC was performed using a Waters Alliance 2795 Alliance HT system (Waters) and an Atlantis dC_{18} column, 3 mm × 100 mm, 5 μm (Waters) maintained at 35 °C. The column was eluted isocratically with 0.1% aqueous formic acid:methanol (90:10) at 0.2 mL/min. As is the standard practice when analysing a crudely prepared matrix, the column eluent was initially diverted to waste until 1.2 min after injection when it was switched to deliver the eluent to the mass spectrometer; this can reduce unnecessary instrument maintenance. After the elution of the analytes, the column was washed with methanol:0.1% aqueous formic acid (90:10) to remove any remaining endogenous compounds. The injection volume was 20 μL and the time from injection to injection was 11 min. All aspects of system operation and data acquisition were controlled using MassLynx^{TM} NT 4.0 software.

2.5.2. Mass spectrometry

A Quattro *micro* tandem mass spectrometer (Waters) was used for all analyses. Ionisation was achieved using electrospray in the positive ionisation mode (ES+). The optimum conditions were: capillary voltage, 3.5 kV; source block temperature, 120 °C; desolvation gas (nitrogen) heated to 350 °C and delivered at a flow rate of 700 L/h.

Table 1
MRM transitions and conditions for the measurement of GHB, GBL, 1,4-BD and their deuterated internal standards

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
GHB	105	87	10	7
GHB-d6	111	93	10	7
GBL	87	45	25	15
GBL-d6	93	49	25	15
1,4-BD	91	73	12	5

In order to establish the appropriate multiple reaction monitoring (MRM) conditions for the individual compounds, solutions of the standards (5 mg/L, in 0.1% formic acid:methanol (90:10 (v/v)) were infused into the mass spectrometer and the cone voltage (CV) optimised to maximise the intensity of the protonated molecular species $[M + H]^+$. Collision-induced dissociation (CID) of each protonated molecule was performed. The collision gas (argon) pressure was maintained at 5×10^{-3} mBar and the collision energy (eV) adjusted to optimise the signal for the most abundant product ions, which were subsequently used for MRM analysis. Table 1 summarises the MRM transitions and conditions for all analytes and IS (deuterated analogues). Quantification was performed by integration of the area under the specific MRM chromatograms. Drugs were quantified by reference to the integrated area of their respective deuterated analogues. For 1,4-BD, no deuterated analogue was available, thus GHB-d6 was used for internal standardisation. Standard response curves were generated daily for the various analytes in urine using a weighted (1/*x*) least-squares linear regression model. For samples where the response exceeded the upper limit of the standard curve, dilutions (into blank urine) were prepared and the sample reanalysed.

All aspects of data acquisition were controlled using MassLynx^{TM} NT 4.0 software with automated data processing using the QuanLynx^{TM} program (Waters).

2.6. LC–MS/MS assay validation

2.6.1. Linearity, limit of quantification (LOQ), precision and analytical recovery

To assess method linearity, urine calibrators (1–80 mg/L) were prepared for analysis by the dilution method and then analysed using LC–MS/MS.

The LOQ was defined as the concentration of the lowest calibrator which was calculated to be within ±20% of the nominal value and with a % CV less than 20% [26,27].

Precision (within-batch and between-batch) and analytical recovery were evaluated by the preparation and analysis of low and a high QC samples, i.e. 4 and 40 mg/L, respectively.

2.6.2. Stability of prepared samples

Two urine calibrators (10 and 50 mg/mL) were prepared for LC–MS/MS analysis by the dilution method. Each diluted sample was aliquoted into 24 separate vials and placed in the autosampler (maintained at 10 °C) until analysis. For each concentration, an injection was made every hour over a period of 24 h. The stability of the processed samples was tested by regression analysis in which the absolute peak areas of GHB and GBL at these two concentrations were plotted versus injection time. Instability of the processed samples would be indicated by a slope, that was significantly different from zero ($P < 0.05$).

2.6.3. Assay selectivity

To assess the effects of the biological matrix on the detection of the analytes, two types of experiments were performed. In the first experiment, control urine samples were diluted 1:20 with water only and then spiked with a mixed standard solution containing GHB, GBL, 1,4-BD and the deuterated standards at a concentration of 10 mg/L. Following LC–MS/MS analysis (five replicate injections for each matrix), the responses (based on peak area) were determined for each analyte and compared to the responses obtained with the same mixed standard solution spiked into water only. A two-sided *t*-test was used to identify any significant differences ($P < 0.05$).

The second experiment, involved a continuous post-column infusion (at 10 μL/min) of a mixed standard containing GHB, GBL, 1,4-BD and the deuterated standards (at a concentration of 10 mg/L) to produce a constant elevated response in each MRM channel. The interference of this constant signal was monitored following the injection of control diluted (1:20) urine samples into the LC–MS/MS system. Five different blank urines were investigated and their MRM chromatographic profile was compared to the response obtained following the injection of water only.

2.7. Sample analysis by GC/MS

For comparative purposes, urine samples were analysed for GHB based on a routinely used and previously published GC–MS method [21]; this method has a total analysis time of at least 1 h. Briefly, a 20 μL aliquot of urine was mixed with acetonitrile containing the deuterated standard. After centrifugation, the supernatant was evaporated to dryness and a silylation procedure performed. The GC–MS system consisted of a HP6890 autosampler and GC system coupled to a HP5973 mass selective detector (Agilent Technologies). A HP5-MS capillary column (30 m × 0.25 mm; 0.25 μm film thickness) was used. The oven was programmed to rise from an initial temperature of 70 °C which was maintained for 1 min, before ramping to 100 °C (10 °C/min) and then to 295 °C (30 °C/min) which was maintained for a further 1 min. The following ions were monitored in electron impact ionisation mode: *m/z* 233 and *m/z* 204 for GHB and *m/z* 239 for GHB-d6. The LOQ was 1 mg/L for GHB.

3. Results and discussion

The product ion spectra obtained with the pure standards are shown in Fig. 1. For GHB, the maximum sensitivity was achieved by monitoring the fragmentation of the protonated molecular species, i.e. *m/z* 105 to a product ion of *m/z* 87; this corresponded to the loss of a water molecule in the collision cell under controlled conditions. However, full scan analysis of GHB indicated that, under some conditions, the molecule might also lose water within the instrument source, effectively resulting in the generation of $[GHB\text{-}H_2O]^+$. Thus, in order to differentiate between this and *actual* GBL, it was necessary to achieve complete chromatographic separation of GHB and GBL. This was accomplished using an Atlantis dC_{18} column; these columns are a difunctionally bonded, silica-based line of reversed-phase C_{18} column and are designed specifically for the retention of polar compounds in aqueous mobile phases. The applied chromatographic conditions ensured elution of all of the analytes of interest and produced chromatographic peaks of acceptable symmetry. The $[GHB–H_2O]^+$ produced as a result of in-source fragmentation of GHB eluted at 4.5 min and was clearly separated from GBL which eluted at 5.4 min. Fig. 2 shows the

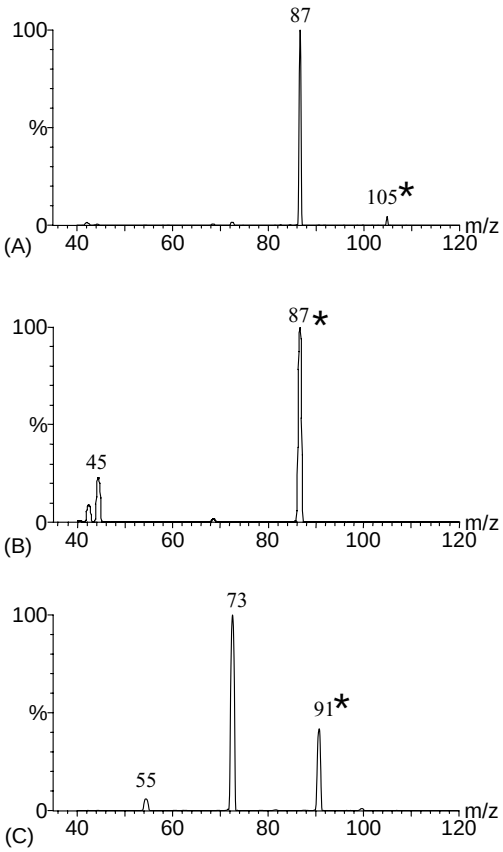

Fig. 1. Product ion spectra for GHB (A), GBL (B) and 1,4-BD (C). Pure standards (5 mg/L) were infused into the mass spectrometer and the cone voltage (CV) optimised for the precursor ion (∗). CID was then performed and product ion spectra acquired under optimum conditions for the most abundant product ion.

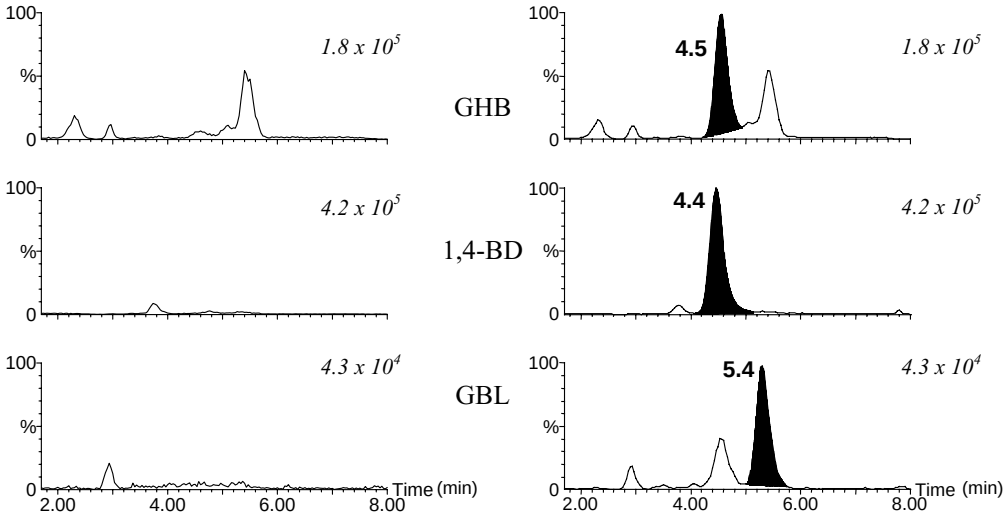

Fig. 2. MRM chromatograms obtained with a single injection of a control urine sample prepared by the dilution method (left-hand column) and the same sample enriched with 10 mg/L of GHB, GBL and 1,4-BD (right-hand column). Peak intensity is shown in the top right-hand corner of each trace.

MRM chromatograms obtained following analysis of a control urine sample and the same sample enriched with GHB, GBL and 1,4-BD.

Initially, two methods of sample preparation, i.e. dilution only and a sample clean-up method using OASIS cartridges were investigated and the sensitivity (based on signal-to-noise (S:N) ratio) for the calibrators compared. The dilution only method demonstrated sufficient sensitivity to detect all three analytes at 1 mg/L in urine. The cartridge clean-up led to an increase in sensitivity of ∼2-fold for GHB and GBL and ∼3-fold for 1,4-BD. Since the simpler procedure already enabled the measurement of endogenous urinary levels of GHB, no further attempts were made to optimise the cartridge procedure and all subsequent analyses and method validation used the simpler, dilution method. Although the cartridge method did not appear to be particularly advantageous in the case of the analysis of urine, it may prove useful for the analysis of alternative specimens where sample may be limited or additional sensitivity may be required, e.g. hair samples.

Calibration curves were constructed for each compound by plotting the peak area ratios (compound/internal standard) against the concentration. In each case, a weighted ($1/x$) linear regression line was applied. Linear responses were obtained for GHB and 1,4-BD over the range investigated (1–80 mg/L). GBL produced a linear response over the range 1–50 mg/L. The linearity data for the analytes in urine is summarised in Table 2. For each analyte, the lowest calibrator, i.e. 1 mg/L, satisfied the criteria for LOQ. Within-batch and between-batch precisions were highly satisfactory with all coefficients of variation (CVs) less than 7%. Analytical recoveries ranged from 90 to 107% (Table 3).

The in vitro inter-conversion between GHB and its lactone (GBL) is well documented and can be influenced by factors such as time, temperature and pH [24]. Consequently, in the development of any analytical technique, the potential for any undesired instability or inter-conversion must be assessed. To this end, the stability of prepared samples was investigated by repeated injections of two calibrators, i.e. 10 and 50 mg/L, over a period of 24 h. For both concentrations of GHB, and the low concentration of GBL, there was no significant instability over the course of the experiment. At the higher concentration of GBL however, a slight negative slope was observed; regression analysis indicated that this was significantly different from zero ($P < 0.05$) (Fig. 3). This apparent instability is likely to be as a result of a mild degradation of the analyte, rather than being indicative of conversion of the lactone to GHB, since there was

Table 2
Linearity and sensitivity of GHB, GBL and 1,4-BD in urine

Linearity data					Sensitivity data
Compound	Slope[a]	Intercept[a]	CV of slope (% over 5 consecutive days)	r^2 (range over 5 consecutive days)	LOQ (mg/L)
GHB	0.0469	0.0246	5.4	0.997–0.999	1.0
GBL	0.1785	0.0199	4.5	0.998–0.999	1.0
1,4-BD	0.1244	−0.0410	6.6	0.996–0.999	1.0

Samples were prepared by the dilution method as described in the text.
[a] Reported values are the mean of five determinations over 5 consecutive days

Table 3
Precision[a] and analytical recovery data for GHB and its precursors in urine.

Compound	Low QC (4 mg/L)			High QC (40 mg/L)		
	Mean (mg/L)	Recovery (%)	CV (%)	Mean (mg/L)	Recovery (%)	CV (%)
Within-batch precision						
GHB	3.9	98	3.2	42.7	107	3.5
GBL	3.7	93	3.2	36.1	90	2.9
1,4-BD	4.0	100	2.2	40.0	100	3.1
Between-batch precision						
GHB	4.1	103	5.3	40.0	40.0	3.4
GBL	4.0	100	6.6	39.8	39.8	6.3
1,4-BD	3.9	98	3.8	40.5	40.5	4.7

[a] Within-batch precision was evaluated by the preparation and analysis of five replicates of the low and high QC in a single assay. Between-batch precision was evaluated by the preparation and analysis of each QC over 5 consecutive days.

no concurrent increase in the absolute peak area for the latter analyte.

In the absence of rigorous sample preparation, the components of any biological matrix will almost certainly have an effect on the detection of the analyte [28,29]. To assess this, we compared peak area responses in both the presence and absence of diluted urine. In these experiments, the presence of diluted urine had no significant effect on peak responses for GBL or 1,4-BD. However, for GHB the mean peak area was reduced by 11% in the presence of one (out of the five urine samples investigated) in comparison to the response obtained in the absence of biological sample ($P < 0.05$). The mean peak area for the internal standard, i.e. GHB-d6 was also reduced by a similar degree in this sample. These results emphasise the importance of internal standardisation; under normal assay conditions, the response for the analyte was expressed relative to its respective deuterated analogue. Since the analogue has the same physico-chemical properties as the unlabelled analyte, it will undergo suppression of ionisation (or indeed enhancement) to the same extent and the ratio between them will remain the same, thus compensating for any effect due to the matrix.

Post-column infusion experiments (based on the method described by Bonfiglio et al. [28]) were performed to provide information of the effect of matrix throughout the course of the *whole* chromatographic run, i.e. not just at the elution time for the analytes. The effect on GHB response obtained following the injection of a water-only control is shown in Fig. 4A. The observed changes in response corresponded to time of the column washing procedure step (after 6 min). The

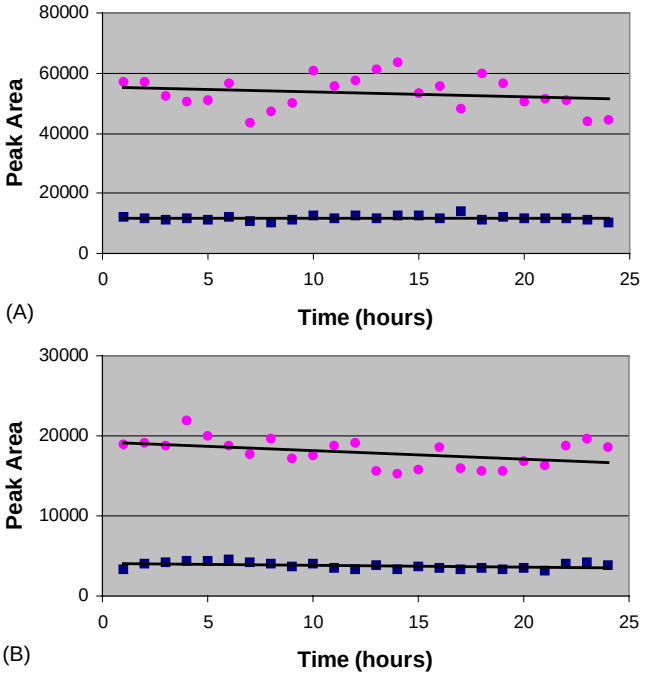

Fig. 3. Analysis of urine enriched with 10 mg/L (squares) and 50 mg/L (circles) of GHB (A) and GBL (B). Absolute peak areas are plotted over a 24-h period.

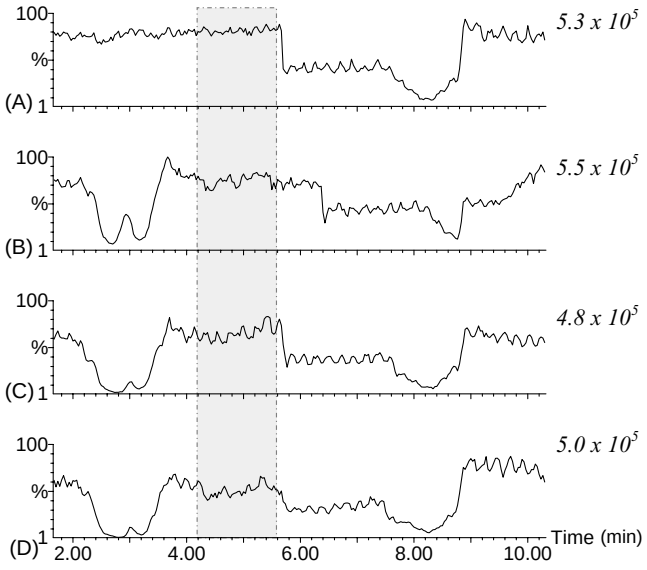

Fig. 4. Evaluation of the matrix effect on GHB response of an injection of a water-only control (A), a diluted control urine (B) and two authentic urine samples that were negative for GHB (C and D). The shaded area indicates the elution positions of GHB and its precursors. Peak intensity for GHB is shown in the top *right*-hand corner of each trace.

effects on GHB response obtained following the injection of several diluted urine samples are also included (Fig. 4B and D). The main effect was a reduction in response observed between ~2.3 and 3.6 min. It is likely that this effect is due to the elution of endogenous components. However, this apparent suppression diminished and the normal level of response was restored by the elution time of GHB, GBL and 1,4-BD.

The potential interference in GHB quantification by its isomers, i.e. alpha and beta-hydroxybutyric acid, was assessed since they are also naturally occurring compounds; alpha-hydroxybutyric acid is a fruit acid found in apples and apple juice and beta-hydroxybutyric acid is a natural by-product of "fasting" human metabolism. The latter isomer is known to be present in urine and can be elevated in the urine of alcoholics [30]. To evaluate the potential for interference, standards were analysed using the developed LC–MS/MS method. Beta-hydroxybutyric acid eluted at 5.1 min and was chromatographically resolved from GHB. In contrast, alpha-hydroxybutyric acid did not produce any response in the monitored MRM channel. Further investigations (by performing infusion experiments) revealed that alpha-hydroxybutyric acid did not form the protonated species under the conditions used in the LC–MS/MS method; for this compound the predominant species was sodiated species, i.e. $[M + Na]^+$. Therefore, in order to determine the retention time for this particular isomer, LC–MS was performed and both the protonated molecule and the sodiated species were monitored, i.e. *m/z* 105 and 127, respectively. Alpha-hydroxybutyric acid was demonstated to elute at 5.9 min and was chromatographically resolved from GHB. Fig. 5 shows the summed ion chromatograms obtained following LC–MS analysis of alpha, beta and gamma-hydroxybutyric acid.

The validated LC–MS/MS method was applied to the analysis of 182 authentic urine samples. These urine samples were collected from club-goers attending a post dance-club 'chill-out' venue and were the result of two separate raids by the Belgian Police Department. Seven samples contained GHB at concentrations above or equal to 2 mg/L. The same seven samples were independently confirmed by the GC–MS procedure. Only two of these samples were above the recommended interpretative cut-off value for urine, i.e. 10 mg/L, and were 956 and 1411 mg/L, respectively. These concentrations would be indicative of exogenous ingestion of GHB and/or the precursors and are within the range of those reported in other cases of possible GHB and/or precursor ingestion, e.g. Couper reported urinary GHB concentrations of 1600 and 2200 mg/L in two non-fatal overdose cases [31]. Bosman and Lusthof [32] reported concentrations ranging from 14 to 2000 mg/L in urine samples analysed in cases of suspected drugging and driving under the influence. LeBeau et al. [20] reported a urinary GHB concentration of 308 mg/L in the case of a suspected DFSA.

The two samples that contained the high levels of GHB were also found to contain small amounts of GBL, i.e. 0.5 and 2% of the determined GHB concentrations, respectively. None of the authentic urine samples contained 1,4-BD. During the development and application of the analytical procedure, we minimised any potential for inter-conversion of GHB and GBL. Thus, the presence of GBL in these samples may suggest that the source of GHB could be due to the ingestion of drug preparations based on GBL. Although the conversion of the precursors to GHB is rapid, their presence, in both ante- and post-mortem urine, has also been reported by other investigators [19,33,34].

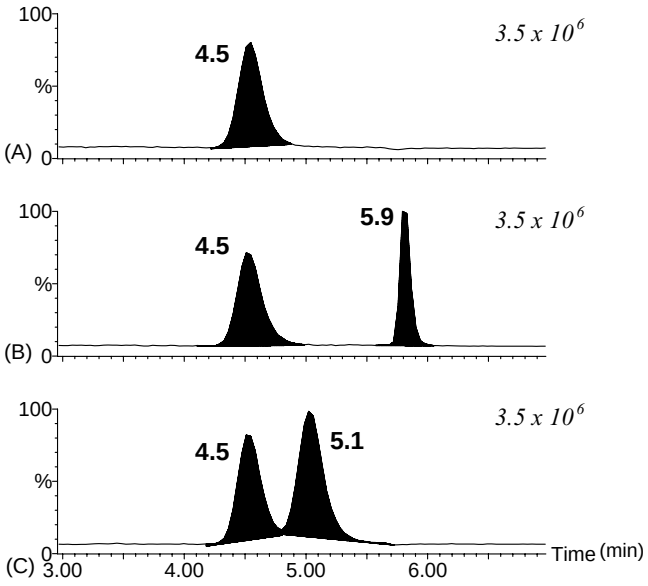

Fig. 5. LC–MS analysis of hydroxybutyric acid isomers. Ion chromatograms obtained following the analysis of gamma-hydroxybutyric acid (GHB) only (A) and GHB in the presence of alpha and beta-hydroxybutyric acid (traces B and C, respectively). Peak intensity is shown in the top *right*-hand corner of each trace and is the sum of the responses obtained for both the protonated and the sodiated species species, i.e. *m/z* 105 + 127.

Whilst the routine FPIA-screening of the authentic urine samples revealed that more than 80% of the samples were positive for one or more illicit drugs, predominantly this involved cannabinoids, amphetamine and/or ecstasy and, to a lesser extent, cocaine metabolites; overall there was a low prevalence of GHB-positives (1.1%).

4. Conclusions

To the very best of our knowledge, the method presented here is the first demonstration of the use of LC–MS/MS for the simultaneous analysis of GHB and its precursors in urine samples. The method offers sufficient sensitivity to enable the measurement of endogenous levels of GHB and to identify exogenous ingestion. Validation experiments have demonstrated the method to be both accurate and robust. The LC–MS/MS results obtained following the analysis of authentic samples correlated with a more labour-intensive GC–MS method.

Although the data presented here indicate that the actual prevalence of GHB-positives might be quite low, the hype and publicity surrounding these drugs has led to a dramatic increase in the number of requests for their analysis in biological samples (and particularly in urine). In the main, this demand has been met by the GC–MS method. However, clearly this technique is not ideally suited for the screening of the large numbers of samples that are currently encountered, being both labour-intensive and time-consuming. The developed LC–MS/MS procedure has comparable sensitivity to the GC–MS method, i.e. LOQ of both techniques is ∼1 mg/L however, it offers several advantages over the latter. It enables the simultaneous quantification of the GHB *and* the precursors in a single analysis; this can facilitate the identification of the chemical basis of any seized putative drug preparations or, if present in the biological specimen, can provide information of the chemical nature of the ingested drug. One of the main advantages of this procedure, is that it involves far fewer manipulations and, with a total analysis time of ∼12 min compared to ∼1 h for GC–MS, is also less time-consuming.

Clearly, the simplicity and speed of the described LC–MS/MS technique should prove a useful screening tool and, with putative positive samples confirmed by an additional assay, is ideal to meet the current increased analytical demand on laboratories.

To date we have not had the opportunity to apply this method to the analysis of post-mortem samples or any alternative specimens, e.g. hair, however future experiments on this topic are planned.

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