Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 6, pp. 483–487, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Therapeutic gamma-hydroxybutyric acid monitoring in plasma and urine by gas chromatography–mass spectrometry

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Abstract: A gas chromatographic–mass spectrometric (GC–MS) method for the determination of therapeutic levels of gamma-hydroxybutyric acid (GHB) in plasma and urine samples is described. GHB is converted to its lactonic form gamma-butyrolactone (GBL) which is extracted from biological fluids after the addition of the internal standard delta-valerolactone. Final GC–MS analysis is obtained under electron impact selected ion monitoring (SIM) conditions. Mean relative recoveries of GHB from plasma and urine are 75.5% (RSD% = 2.2) and 76.4% (RSD% = 2.4), respectively. The assay is linear over a plasma GHB range of 2–200 $\mu g\ ml^{-1}$ ($r = 0.999$) and a urine GHB range of 2–150 $\mu g\ ml^{-1}$ ($r = 0.998$). Intra- and inter-assay relative standard deviations ($n = 5$) determined at 10 and 100 $\mu g\ ml^{-1}$ are below 5%. The method is simple, specific and accurate, and may be applied for analytical purposes related to pharmacokinetic studies and therapeutic drug monitoring.

Keywords: *Gamma-hydroxybutyric acid; therapeutic drug monitoring; GC–MS.*

Introduction

Gamma-hydroxybutyric acid (GHB) is an endogenous constituent of mammalian brain and is considered to function as a neurotransmitter or neuromodulator [1].

GHB has been used as an intravenous anaesthetic agent [2] and in the treatment of sleep disorders [3] because of its neuropharmacological effects when administered to man. It has also been introduced into the treatment of alcohol withdrawal syndrome and alcohol dependence in man [4, 5]. More recently, the first reported cases of abuse of GHB have appeared in the USA [6].

Pharmacokinetic studies have been carried out in animals [7–10] and human alcohol dependents [11]. After single and repeated therapeutic oral doses (25 mg kg^{-1} every 12 h) GHB is readily absorbed, rapidly eliminated and urinary recovery of unchanged GHB is negligible [11]. The multiple-dose regimen results neither in accumulation of GHB nor in time-dependent modification of its pharmacokinetics. Doubling of the dose (50 mg kg^{-1}) results in increases in t_{max}, C_{max}, dose-normalized AUC, terminal half-lives ($t_{1/2z}$) and mean residence time [11].

Because of the possibility of interconversion of GHB into its lactonic form gamma-butyrolactone (GBL) under certain chemical conditions or *in vivo* by enzymatic conversion, it was previously thought that GBL could be consistently present in biological fluids of animals or humans together with GHB, even after GHB administration [12–14]. However, subsequent studies failed to detect significant levels of lactone in blood, plasma [15–17] and tissue samples [18].

Up to now several analytical methods have been reported to detect the presence of GHB in biological fluids or tissues of animals or human subjects. These determinations were made to detect GHB as an endogenous substance or after its administration. In the last twenty years, various gas chromatographic (GC) methods have been employed which may be differentiated with regard to the treatment of the biological sample. In many methods the procedure involves the conversion of GHB to GBL by heating at 80–100°C in the presence of a strong mineral acid and subsequent ex-

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traction of GBL and its detection, with or without derivatization [10, 15, 17–22]. In other methods [23–25] the species extracted from biological fluids is the original GHB, under conditions avoiding the incidental formation of GBL. GHB is then derivatized for efficient GC analysis.

The aim of this study was to develop a specific and accurate analytical method to detect therapeutic levels of GHB in human plasma and urine samples. In order to overcome the relative lack of selectivity shown by universal GC detectors, a gas chromatographic–mass spectrometric (GC–MS) method, using the selected ion monitoring (SIM) technique, was applied. The described procedure also involves the conversion of GHB to GBL prior to extraction and final detection without derivatization, in order to avoid excessively complex preparation of samples.

Experimental

Chemicals

Methanol and benzene of analytical grade were obtained from Merck (Darmstadt, Germany). Potassium phosphate monobasic, sodium phosphate dibasic dihydrate (used for the 1.5 M, pH 6.5 phosphate buffer) and sodium chloride were obtained from Merck. Sodium hydroxide, hydrochloric acid and perchloric acid (Merck) were prepared as 5, 6 and 0.8 N solutions in distilled water, respectively. A stock solution of gamma-hydroxybutyric acid sodium salt (Aldrich-Chemie, Steinheim, Germany) was prepared in methanol at a concentration of 1 mg ml^{-1} (as acid equivalent). Subsequent working solutions for calibration samples were prepared by appropriate dilutions of the stock solution. A standard solution of delta-valerolactone (internal standard, I.S.) (Aldrich-Chemie) was prepared in benzene at a concentration of 0.15 mg ml^{-1}.

Preparation of samples

Plasma and urine samples (n = 250 and 100, respectively) were collected from 50 patients attending the Addiction Medicine Unit, ULSS 21-Padova, treated with 25–50 mg kg^{-1} of GHB every 12 h for alcohol withdrawal syndrome and alcohol dependence, at 0–60 min (plasma) and at 0–4 h (urine) after the daily GHB dose. Samples were stored at −40°C for 1 day prior to assay.

Plasma samples (2 ml) were deproteinized with 2 ml of cold 0.8 N perchloric acid, and precipitates were removed by centrifugation for 10 min at 4500 rpm. Urine samples (2 ml) were acidified with 0.2 ml of 6 N hydrochloric acid, and the mixtures, together with 3 ml of protein-free supernatants obtained from plasma samples, were heated at 80°C for 20 min in order to convert GHB to the lactone form. A 300 mg mass of sodium chloride was added to the cooled solutions and their pH subsequently adjusted by first adding 1 ml of 1.5 N phosphate buffer (pH 6.5) and then adding 5 N sodium hydroxide dropwise to bring the pH to 6.5. A 0.2 ml volume of the 0.15 mg ml^{-1} delta-valerolactone solution was added and the samples were extracted with a fixed volume of benzene (6 ml for urine samples and 8 ml for plasma samples). After centrifugation for 10 min at 4500 rpm, 5 ml and 6.5 ml of the organic phase, respectively, for urine and plasma samples, were removed and concentrated to a final volume of 0.2 ml by gently blowing nitrogen over the samples maintained at 35°C in a water bath. Aliquots (3-μl) of the final solutions were injected into the gas chromatograph for GC–MS analysis.

Calibration samples were prepared by spiking human urine and plasma with appropriate amounts of GHB in the range 0–150 μg ml^{-1} for urine and 0–200 μg ml^{-1} for plasma. These samples were processed according to the above procedure. Calibration curves were constructed by plotting the peak area ratios (total ion GBL/total ion I.S.) versus GHB concentration. Equations for the curves were calculated by linear regression analysis, using at least five calibration points per curve (corresponding to GHB concentrations of 2, 5, 50, 100 and 200 μg ml^{-1} for plasma and 2, 5, 20, 50 and 150 μg ml^{-1} for urine).

Instrumentation and analytical conditions

GC–MS analyses were performed on a Hewlett–Packard 5790 A gas chromatograph coupled to a Hewlett–Packard 5970 A mass selective detector (MSD). Separation was achieved with a Hewlett–Packard ULTRA 1 bonded phase capillary column (12 m × 0.20 mm) with a 0.33-μm film thickness, connected to the MSD through a direct capillary interface. The injector port was a capillary split injector with a split silanized glass insert. The inlet pressure of the carrier gas (He) was 0.2 kg cm^{-2} and a split ratio of 10:1 was used.

Injector and interface temperatures were 250 and 275°C, respectively. The oven was initially held at 50°C for 0.6 min, then programmed at 15°C min^{-1} to a final temperature of 275°C. The MSD was used in the electron impact selected ion monitoring (SIM) mode, programmed to detect characteristic ion species at *m/z* 41, 42, 56, 86 and 100 for GBL and delta-valerolactone. The electron multiplier was set at 400 V above the autotune voltage.

Results

Some experiments were performed in order to evaluate the possible *in vivo* formation of GBL, the lactone form of GHB. Plasma and urine samples containing considerable levels of GHB (from 80 to 100 μg ml^{-1}) were extracted according to the experimental procedure but omitting the acidification and heating steps. In both plasma and urine no GBL peaks could be detected by GC–MS analysis.

Further experiments were carried out to evaluate the incidental formation of GBL under the particular physico-chemical conditions characteristic of the biological fluids under study. In particular, some blank urine samples adjusted to pHs from 5 to 7 and spiked with 50 μg ml^{-1} of GHB were allowed to stand at room temperature for 3 days. The samples were then extracted and analysed as reported in the Experimental section but omitting the acidification and heating steps. In no cases could any GBL be detected.

The conversion of GHB to GBL in plasma and urine samples was studied and consequently optimized for the best reaction yields. Three parameters were particularly considered: acidification, reaction temperature and reaction time. Experiments were performed on water and urine samples, using concentrated sulphuric acid and 6 N hydrochloric acid at temperatures ranging from ambient to 100°C and reaction times from 5 to 30 min. The best reaction yield was reached by using 6 N hydrochloric acid, at a temperature of 80°C and a minimum reaction time of 20 min. As deproteinizing agents for plasma samples, cold 20% trichloroacetic acid and cold 0.8 N perchloric acid were tried. The latter was more efficient and also gave good acidic conditions permitting effective GHB conversion (at 80°C for 20 min) without the addition of 6 N hydrochloric acid.

Attempts at extracting at acidic pHs (e.g. pH 1) were ineffective, and the extraction step was consequently optimized at neutral pH (6–7).

During the final evaporation of the organic extract it was found necessary to use a low nitrogen flow and a maximum temperature of 35°C. Higher temperatures caused unacceptable losses of both GBL and I.S.

When the described method was employed the mean relative recoveries of GHB from plasma and urine were found to be 75.5% (RSD% 2.2) and 76.4% (RSD% 2.4), respectively.

A typical chromatogram obtained from the GC–MS analysis of an authentic plasma sample extract is shown in Fig. 1. Similar traces were obtained for urine samples. With this method no interfering substances were detected in 250 plasma or 100 urine samples. The retention times of GBL and delta-valerolactone under the employed gas chromatographic conditions were 2.6 and 4.0 min, respectively (RRT = 0.65 ± 0.015 SD).

By linear regression, straight lines can be described by the following equations: $y = 0.0171x - 0.0240$ for plasma, and $y = 0.0220x - 0.0407$ for urine. The correlation coefficients of these lines were 0.999 and 0.998, respectively.

The within-day relative standard deviations (RSDs) were evaluated by analysing five aliquots of supplemented plasma and urine samples at 10 and 100 μg ml^{-1} concentrations. Day-to-day RSDs were determined on five different days by construction of a complete standard curve and daily analysis of two 10 and 100 μg ml^{-1} aliquots of GHB supplemented plasma and urine samples. All these data, as well as those of the linear regression analysis, are reported in Table 1.

The lowest plasma and urine concentrations of GHB that could be measured, with a signal-to-noise ratio of 5, were 0.2 μg ml^{-1} and 0.1 μg ml^{-1}, respectively. This means that, even though the linearity of the present method was investigated to assay therapeutic GHB levels [11], quantitation of GHB in plasma and urine at subtherapeutic levels is possible and could be more accurately achieved by obtaining suitable calibration curves.

In the SIM mode five different ion species at *m/z* 41, 42, 56, 86 and 100 were monitored in five separate channels being the most abundant and characteristic for GBL (*m/z* 41, 42, 56, 86)

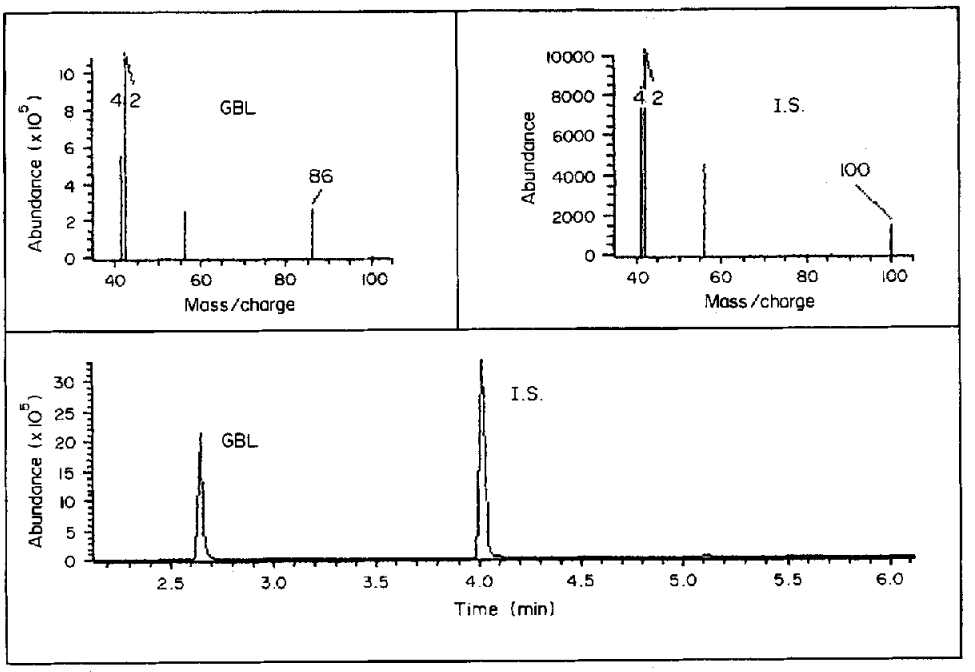

Figure 1
GC–MS (SIM) analysis of an authentic plasma sample containing 35.5 μg ml^{-1} of GHB. Mass-chromatogram and SIM-mass spectra of GBL and I.S., respectively, are shown in lower and upper parts of figure.

Table 1
Calibration curves and reproducibility data for determination of GHB in plasma and urine samples

Sample	Linear regression equation	Conc. range (μg ml^{-1})	Correlation coefficient	RSD Within-day μg ml^{-1} 10	RSD Within-day μg ml^{-1} 100	RSD Between-day μg ml^{-1} 10	RSD Between-day μg ml^{-1} 100
Plasma	$y = 0.0171x - 0.0240$	2–200	0.999	3.5	4.1	4.3	4.6
Urine	$y = 0.0220x - 0.040$	2–150	0.998	4.5	3.9	4.9	4.8

y = Peak area ratio (total ion, SIM).
x = Concentration (μg ml^{-1}).

and the I.S. (*m/z* 41, 42, 56, 100). As long as all GC–MS parameters remained constant, area ratios for the ion species of GBL (86:56, 86:42, 86:41) and the I.S. (100:56, 100:42, 100:41) in all test samples and standards remained the same. In a typical batch analysis of urine samples spiked with GHB (2–150 μg ml^{-1}) and I.S. (30 μg total), the average area ratios for the above ion pairs are those reported in Table 2.

Lastly, plasma samples collected from patients treated with daily GHB doses of 25 and 50 mg kg^{-1} showed mean peak concentrations of 55 (range 24–88) and 90 (range 51–158) μg ml^{-1}, respectively.

Table 2
Average area ratios of selected ion pairs obtained in a typical batch analysis of spiked urine samples

GBL Ion pair	GBL Area ratio* mean (n = 5)	I.S. Ion pair	I.S. Area ratio* mean (n = 5)
86:56	1.0451	100:56	0.3965
86:42	0.2346	100:42	0.1866
86:41	0.4119	100:41	0.2137

*RSD% <5 in all determinations.

Discussion

The described GC–MS (SIM) method is an effective analytical tool for quantitative analysis of both plasma and urine samples containing therapeutic levels of GHB.

Experiments to reveal the possible presence of original GBL in the analysed biological samples (due to both enzymatic and chemical formation) or to incidental formation during storage of samples (see 'Results' section) demonstrate: (a) the sole presence of GHB in plasma and urine samples after GHB intake, according to previous reports for blood, plasma [15–17] and tissue samples [18]; and (b) GBL is not formed even when samples remain at room temperature for relatively long periods. This means that the present method allows accurate quantitative analysis of GHB as GBL.

Experiments regarding the extraction pH confirm the report of Lettieri and Fung [17]: in acidic conditions the lactone moiety of the analyte under study may be protonated, leading to poor recoveries. Hence, efficient extractions necessitate pH values of 6–7.

The well-known intrinsic selectivity of GC–MS (especially when using the SIM acquisition mode) is successfully verified in this analytical context. In fact, although the selected ion species for SIM acquisition have quite low m/z values, the present procedure allows a good level of specificity, sufficient to avoid possible interferences arising from endogenic or therapeutic substances.

The sensitivity of the method appears to be suitable for analytical purposes related to pharmacokinetic studies and therapeutic drug monitoring.

Delta-valerolactone as internal standard is a good alternative to deuterated analogues of GHB, which are expensive and not readily available. A relative disadvantage, due to the relative instability of this I.S., is that it must be added after the heating step and not at the beginning of the extraction procedure. Its corresponding hydroxy-acid, delta-hydroxy-valeric acid, would be a better I.S. (it may be added at the beginning of the extraction procedure) but it is not readily available in commerce.

The linearity and accuracy of the method were good and recoveries of GHB from plasma and urine showed satisfactory values.

Conclusion

The described analytical method proves to be simple, specific and accurate. Hence, it may be used for therapeutic GHB monitoring and for assessment of any time-dependent changes of multiple GHB dosing as applied to alcohol-dependent patients.

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[Received for review 29 July 1992; revised manuscript received 30 November 1992]