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Journal of Chromatography B, 807 (2004) 287–291
JOURNAL OF CHROMATOGRAPHY B
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Liquid chromatographic–mass spectrometric determination of endogenous γ-hydroxybutyrate concentrations in rat brain regions and plasma

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Received 27 January 2004; received in revised form 22 April 2004; accepted 22 April 2004

Available online 25 May 2004

Abstract

A new liquid chromatographic–mass spectrometric (LC–MS) method for determining trace concentrations of γ-hydroxybutyric acid (GHB) in biological samples has been developed. This method utilizes solid-phase extraction for separation, deuterated GHB as an internal standard (IS) and multiple reaction monitoring (MRM) in the negative ion mode to detect the parent and product ions (103 and 57 for GHB, and 109 and 61 for D6-GHB, respectively). The assay produces excellent linearity and reproducibility, with a limit of quantification (LOQ) of about 0.1 μg/ml. The method has been applied for the determination of endogenous GHB in various rat brain regions.

Keyword: γ-Hydroxybutyrate

1. Introduction

γ-Hydroxybutyrate (GHB) is an analogue of the endogenous neurotransmitter, γ-aminobutyric acid (GABA). It is present in various regions of the brain, but its endogenous functions are still incompletely understood. GHB induced release of dopamine and other neurotransmitters in the brain [1,2], and therefore was once thought to play a role in treating Parkinson's disease [3]. In 2002, this compound was approved in the US for treating cataplexy [4]. However, the major use of this molecule (and its analogs) in humans today involves abuse; principally for its alleged hypnotic or euphoric effects, in date-rape, or for inducing muscle building. Some highly intoxicated individuals have died from this drug.

The pharmacokinetics (PK) and pharmacodynamics (PD) of this drug in abuse situations are not well defined. Lettieri and Fung [5] first showed that the elimination of this compound, and its prodrug γ-butyrolactone (GBL), is concentration-dependent, exhibiting non-linear PK. The duration of its effects therefore may not be proportional to the dose administered. The site of action of GHB and its analogs are known to reside in certain regions of the brain, therefore information regarding the PK of GHB in these regions may shed some light on its PD. There is currently no literature reports that describe the relationships between the PK and PD of GHB, using both plasma and brain concentrations.

Existing assay procedures using HPLC and GC–MS may be employed for this purpose. However, the HPLC method, as described recently by de Vriendt et al. [6], lacked sensitivity for the determination of brain concentrations. Various reported GC–MS methods [7–9] have limit of quantification (LOQ) at 0.1–1 μg/ml, but they either require conversion of GHB to GBL, or derivatization to more volatile analytes. These procedures may therefore produce incomplete assay recovery, and interference may potentially occur with substances that can be converted to the eventual analyte(s) under the derivatization conditions.

The present study was aimed at the development of a liquid chromatographic–mass spectrometric (LC–MS) procedure that has the requisite sensitivity for the measurement of endogenous brain and plasma concentrations of GHB, without any chemical derivatization. The procedures were developed using rat brain and plasma samples. While this

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doi:10.1016/j.jchromb.2004.04.022

work was in progress, Borgen et al. [10] reported an LC–MS method for the determination of GHB in humans who have been given therapeutic doses. Details of this procedure are not yet available in the literature, but the stated limit of quantification was 5 μg/ml, using 2-hydroxyvalerate as an internal standard (IS). While this sensitivity is more than sufficient for human PK studies after therapeutic doses, it is about one order of magnitude higher than that reported for GC–MS assays [11].

2. Experimental

2.1. Chemicals and reagents

HPLC grade water was obtained from EM Science (Gibbstown, NJ). Methanol was also of HPLC grade (Fisher Scientific, Fairlawn, NJ). Glacial acetic acid and HPLC grade acetonitrile were obtained from JT Baker (Phillipsburg, NJ). KH_2PO_4 and formic acid, 2-hydroxyisobutyric acid, β-hydroxybutyric acid, and sodium hydroxide (NaOH) were purchased from Aldrich (Milwaukee, WI). *trans*-4-Hydroxycrotonic acid was a product of Tocris (Ellisville, MO). α-Hydroxybutyric acid, γ-aminobutyric acid, NaGHB, and GBL were obtained from Sigma (St. Louis, MO). Deuterated GHB (GHB-D6, Cerilliant, Austin, TX) was used as the internal standard for the LC–MS assay.

2.2. Sample preparation

The internal standard, GHB-D6, was added at an appropriate concentration to the biological sample. Solid phase extraction (SPE) was carried out using Varian Bond Elute SAX cartridges (100 mg, 1 ml, Palo Alto, CA) connected to a Varian Vac Elut 20 manifold and a vacuum pump. Although we have essentially adopted the procedures of de Vriendt et al. [6] for SPE and HPLC, we found that, in our hands, the recovery from this procedure was low and variable, so slight modifications were made in the composition and volume of the eluent used in removing GHB from the SPE column. Our final adopted procedure was as follows: the cartridge was first conditioned serially with 1 ml of methanol, 6 ml of 10% acetic acid, and 1 ml of water at an approximate flow rate of 1 ml/min. An aliquot of 100 μl of the biological sample was placed on the cartridge and a vacuum was applied until the sample was completely pulled into the packing material. The sample was left to interact with the cartridge for 15 min, and then washed with 0.5 ml of water, 0.5 ml of water/methanol (1:1, v/v), and 0.3 ml of methanol, again at a flow rate of 1 ml/min. A strong vacuum was then applied for 10 min to facilitate removal of all solvents. The cartridges were then eluted with 3 ml of 10% acetic acid in acetonitrile. The eluent was applied 300 μl at a time and allowed to pass through the cartridge by gravity. At the end of the process, a gentle vacuum was applied to ensure all eluent was pulled from the cartridge. Each sample was dried under a gentle stream of nitrogen gas and reconstituted in100 μl of HPLC-grade water for LC–MS assay.

2.3. LC–MS conditions

All MS data were collected using a commercially available PE/SCIEX API 3000 triple quadrupole mass spectrometer, equipped with an atmospheric pressure chemical ionization (APCI) heated nebulizer ionization source. The LC portion of the method was carried out with a C_{18} Aqua column, and a Perkin-Elmer Series 200 Autosampler (Perkin-Elmer, Wellesley, MA), employing a mobile phase of 90% 5 mM formic acid and 10% acetonitrile, and a flow rate of 0.9 ml/min. The sample injection volume was 10 μl, and the run time for each sample was 5 min. The nebulizer was set at a temperature of 400 °C, with the curtain gas set at 10 and the nebulizing gas set at 9. The mass spectrometer was set at a declustering potential of −20 eV and collision energy of −20 eV. Multiple reaction monitoring (MRM) was used in the negative ion mode in order to detect the parent ions of mass/charge ratio of 103 and 109 Da, and the product ions of 57 and 61 Da for GHB and IS, respectively. All data were acquired and analyzed using the Analyst® 1.3.1 software (Applied Biosystems, Foster City, CA).

2.4. Examination of potential interfering analytes

In the process of validating this LC–MS method, we tested a variety of endogenous analytes that could possibly pose as interferences to the GHB or IS peaks in our biological samples. Separate solutions of the potential interfering analyte were prepared at 78.7 μM in the presence of GHB (7.87 μM), and GHB-D6 (3.79 μM). The effect of the potentially interfering analyte on the apparent peak height ratio (PHR) of GHB versus GHB-D6 was evaluated.

2.5. Aqueous standards and plasma samples

Calibration curves of GHB in diluted plasma (20-fold) were compared to those of aqueous standards, using the SPE and HPLC/MS conditions described. Endogenous concentrations of GHB in 22 Sprague–Dawley rats were determined using aqueous standards.

2.6. Brain samples

Whole rat brains were isolated and immediately stored in the freezer at −20 °C and thawed before the homogenization process. The thawed brain tissue was weighed and placed in a plastic test tube. Homogenization was performed in the presence of 67.0 mM KH_2PO_4 buffer at 2 ml/g of tissue with a Kinematica Polytron model PT 10/35 homogenizer (Kinematica, Lucerne, Switzerland), and was carried out on ice six times, each time for 10 s at a speed setting of 5 on the instrument. The homogenate was then transferred into microcentrifuge tubes and spun down at 16,000 × *g* at 10 °C

for 10 min to separate out the pellet. SPE would then be carried out on the supernatant fraction.

A recovery study was conducted using radioactive GHB: 0.2 μCi of ^{3}H-GHB was added to triplicate samples of three concentrations of unlabeled NaGHB, in separate tubes, to reach total NaGHB amounts of 0.49, 0.99, and 1.48 μmol. The tubes were then spiked with 300 μl of the homogenized rat brain tissue. These samples were shaken at room temperature at 260 rpm for 15 min to ensure mixing of the contents, and then centrifuged at 13,000 rpm for 10 min. An aliquot of 50 μl of the supernatant was removed from each sample, and the pellet was dissolved in Triton X detergent (1 ml per sample) overnight before scintillation counting.

A standard curve was created from the brains taken from three untreated male Sprague–Dawley rats. The brains were homogenized, and 6–7 NaGHB spiked samples were prepared from each brain. Samples were made by combining 325 μl of the brain homogenate with 125 μl of GHB solution and 50 μl of IS. The concentration of NaGHB in these homogenates ranged from 1.57 to 39.4 μM. The samples were shaken at 260 rpm for 15 min and then centrifuged at 13,000 rpm for 10 min. A volume of 100 μl of the supernatant was subjected to SPE. Each sample was run three times on the LC–MS, and the average PHR was used. The endogenous concentration of GHB was estimated from the standard curve, after accounting for the dilution of the brain homogenate.

Five other rat brains were isolated and separated over ice on filter paper which was coated with ice cold normal saline solution. The brains were separated into the following selected regions: cerebellum, hypothalamus/thalamus, hippocampus, and cortex, and stored in liquid nitrogen immediately. The brain regions were then pooled for homogenization using 67 mM KH_2PO_4 buffer at about 2 ml/g for the cerebellum and cortex, and 1 ml for samples comprising of hippocampus or hypothalamus/thalamus. The homogenates were centrifuged at about 35,000 × *g* at 4 °C for 20 min, and the supernatant fluids separated. The supernatants were then divided into different aliquots and known amounts of GHB and GHB-D6 added to them. Control (blank) samples did not receive added GHB. The samples were then processed by SPE and analyzed by LC–MS as described.

3. Results and discussion

Using our modified HPLC method and UV detection, we were unable to detect any whole rat brain GHB concentrations at the regaining of righting reflex after animals were dosed with 0.67 mg/kg of GBL subcutaneously. In other studies, we have found that this dosage regimen provided measurable GHB concentrations in plasma. Because it was unknown whether our brain processing procedure was effective in extracting GHB into the supernatent phase, we conducted recovery studies with radioactive GHB. Using total GHB amounts of 0.49, 0.99, and 1.48 μmol, in the presence of ^{3}H-GHB, we found that 0.42 ± 0.02 (85.7%), 0.85 ± 0.10 (85.8%), and 1.17 ± 0.01 (79.1%) μmol of GHB were recovered respectively in the separated supernatant. Thus, the lack of measurable brain concentrations of GHB by UV detection was likely due to an insufficiently sensitive assay rather than the lack of extraction of GHB from the brain tissue.

The LC–MS offers a possible approach to improve both the sensitivity and the precision of the assay, since deuterated GHB (GHB-D6), which is commercially available, can be readily used as an internal standard. Through empirical trials, a mobile phase consisting of 90% 5 mM formic acid and 10% acetonitrile was chosen. Under the described MS conditions, GHB produced a significant fragment of *m*/*e* ratio of 57 (parent 103). Selective ion monitoring at *m*/*e* of 57, against an internal standard fragment of *m*/*e* of 61 (parent 109.2), was therefore chosen for quantitation of the analyte concentration. A sample chromatogram obtained from one of our extracted brain samples (Fig. 1) shows that the two peaks could be separately monitored. In this particular brain sample, two peaks at retention times of 3.12 and 3.37 min were also seen. This observation suggests that these peaks are derived from compounds with parent *m*/*e* of 103 and a daughter *m*/*e* of 57. These compounds were later tentatively identified as 2-hydroxyisobutyric acid and α-hydroxybutyric acid, respectively (see Table 1 later). The same two peaks are also seen in rat plasma samples (data not shown).

The apparent loss of two deuterium atoms in the internal standard upon fragmentation suggests that one of the $-CD_2$ units was lost from the molecule. A formula of C_3HD_4O is consistent with the *m*/*e* ratio of 61, suggesting that the *m*/*e* fragment of 57 for GHB may have the formula of C_3H_5O. The possible fragmentation pathways for both GHB and GHB-D6 are shown in Fig. 2. Further studies are needed to determine the exact chemical structure of these fragments.

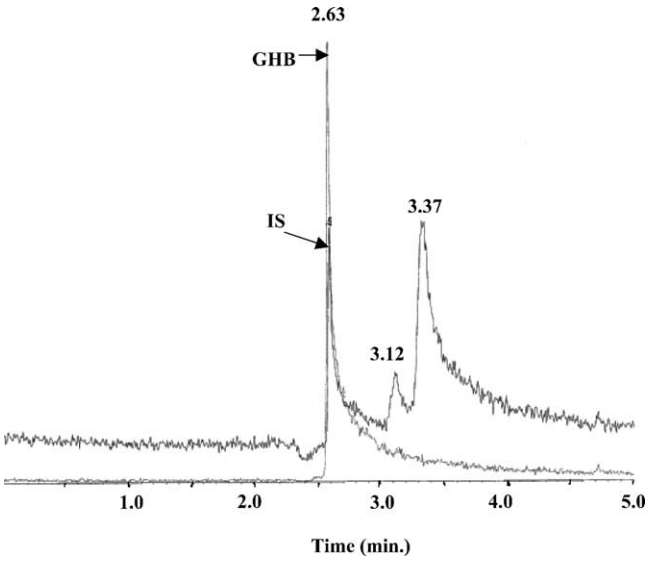

Fig. 1. LC-mass spectrum of a representative brain sample of GHB and GHB-D6.

Table 1
Lack of interference by potentially interfering substances in the LC–MS assay of GHB ($n = 4$ determinations in each experiment)

Potential interfering substance	Average PHR (NaGHB/IS)	P value[a]	Retention time (min)[b]
None	1.78 ± 0.08	–	
β-Hydroxybutyric acid	1.85 ± 0.12	0.24	
γ-Aminobutyric acid	1.75 ± 0.11	0.37	
α-Hydroxybutyric acid	1.84 ± 0.10	0.15	3.37
1,4-Butanediol	1.70 ± 0.12	0.22	
2-Hydroxyisobutyric acid	1.81 ± 0.25	0.43	3.12
2-Hydroxycrotonic acid	1.79 ± 0.08	0.47	

[a] When compared to samples without added interfering substance.
[b] When different from that of GHB.

The possible assay interference by analogs of GHB was examined. Potential interfering substances (X) were added to solutions of GHB and GHB-D6, at a 10-fold molar excess, i.e., ratio of X:GHB = 10:1, and the apparent peak height ratio of GHB/IS was determined. Table 1 shows that, in the presence of β-hydroxybutyric acid, α-hydroxybutyric acid, γ-aminobutyric acid, 1,4-butanediol, 2-hydroxyisobutyric acid, 2-hydroxycrotonic acid, the apparent peak height ratio of GHB to GHB-D6 was not significantly altered. These results indicated that these substances do not interfere with the LC–MS assay of GHB, at least up to a 10-fold excess concentration. In these studies, α-hydroxybutyric acid and 2-hydroxyisobutyric acid showed peaks at 3.37 and 3.12 min, respectively, when a parent *m*/*e* 103 and a daughter *m*/*e* of 57 were monitored.

The quantitative ability of the assay was first established with aqueous standards and plasma samples. Because plasma concentrations in rats after pharmacological doses are quite high, these samples were diluted (with the internal standard solution) at least 10-fold before assay. The diluted plasma standards (after SPE) and aqueous standards

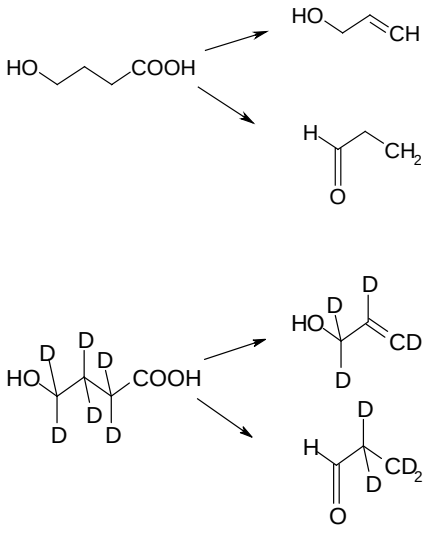

Fig. 2. Possible fragmentation chemistry of GHB and GHB-D6.

produced essentially identical calibration curves, indicating that relative GHB recovery was complete. These standard curves were linear, with an $r^2 > 0.97$. Over a concentration range of 0.79–95.2 μM GHB (internal standard concentration 11.4 μM), nine diluted plasma standards produced a mean intra-day assay variability of 3.6% (range 1.0–6.1%, $n = 3$ per standard), and a mean inter-day assay variability of 9.2% (range 4.6–14.2%, $n = 7$ per standard). The lowest standard used, 0.79 μM in diluted plasma, had an intra-day and inter-day assay variability of 4.4 and 6.5%, respectively. Using this standard as a guide, the limit of quantitation of the present assay can be determined to be 0.1 μg/ml.

Blank samples of rat plasma were then obtained from 22 rats. These samples were then diluted 10-fold with the internal standard solution, and subjected to SPE and LC–MS assay, using the aqueous standards for estimation. The endogenous plasma GHB concentration was determined to be 22.0 ± 1.9 μM.

The LC–MS method was then applied for detecting GHB in brain samples. Known amounts of GHB were spiked into isolated whole brain homogenates from three untreated rats, and a linear calibration curve was obtained ($r^2 = 0.988$). The *x*-intercept allowed us to estimate the endogenous whole brain concentration to be about 7 nmol/g wet weight of rat brain tissue. The variability of spiked samples of GHB into rat brain ranged from 3.7 to 15.9% ($n = 3$ at each concentration), over the same concentration range as the calibration curve. In these studies, the variability was not related to the added concentration of the brain samples.

We next attempted to determine GHB concentrations in the supernatent portions of the homogenates of discrete rat brain regions. Since the wet weight of certain brain regions were quite low, and could not be reliably determined, we normalized GHB concentrations in these samples according to mg of protein, as determined by the Lowry method, using more concentrated reagent concentrations to minimize the reaction volume [12]. Fig. 3 shows the relationship between peak height ratio versus added GHB to the of cortex homogenates, at concentrations up to about 250 pmol/mg protein. The *x*-intercept of this plot provides an estimate of the endogenous concentration of GHB in this preparation, corrected for any possible incomplete recovery. It is seen from Fig. 3 that the endogenous concentration determined was about 100 pmol/mg of protein. Based on an assayed value of about 115 mg protein/g wet weight of brain region for the cortex, this assayed value gave an appropriate equivalent of 10 nmol/g wet weight, quite consistent with the value we obtained earlier using whole brain homogenates (7 nmol/g wet weight).

The endogenous GHB concentrations for the cerebellum, hippocampus and hypothalamus/thalamus regions were determined to be 90.9±21.8, 693±338, and 179±64 pmol/mg protein, respectively (n = 5–6, mean ± S.E.M.). These values were qualitatively consistent with those reported by Eli and Cattabeni [13] for the same male Sprague–Dawley rats whose brain regions were obtained by decapitation

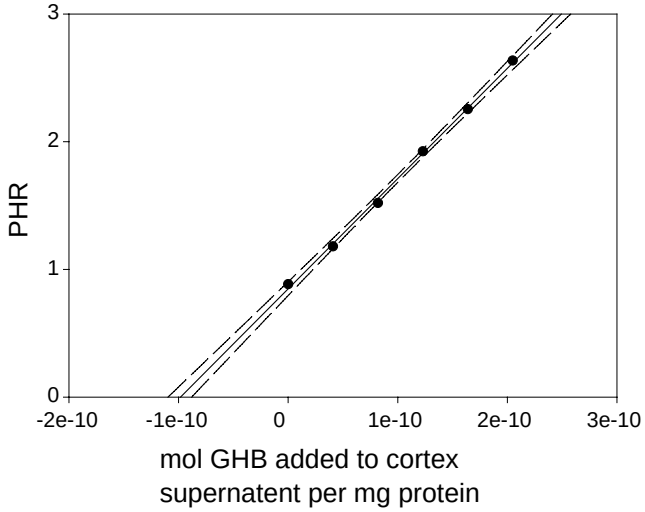

Fig. 3. Standard curve of GHB in the supernatant fraction of the rat brain cortex, from LC–MS determinations.

(2–6 nmol/g wet weight), but these authors did not determine the respective protein concentrations. On the other hand, both the present data and those of Eli and Cattabeni were substantially higher than those found by Vayer et al. [14], who reported concentrations of 4–30 pmol/mg protein, which were about one order of magnitude lower. However, there are significant differences in the methodologies used. For example, our study employed male Sprague–Dawley rats, a LC–MS assay methodology and no derivatization. On the other hand, Vayer et al. [14] used male Wistar rats, a GC–MS assay methodology and derivatization with pentafluorobenzyl bromide and *N*-*t*-butydimethylsilyl-*N*-methyltrifluoroacetamide. In addition, the current study employed an addition method in which the samples are spiked with known amounts of GHB, which avoided the need to correct for assay recovery, either due to incomplete extraction or derivatization. The use of multiple spiked samples and statistical regression to obtain the endogenous GHB values should enhance the reliability of the estimates.

In preliminary studies, we demonstrate that it is also possible to employ this LC–MS method to detect GHB in rat cerebrospinal fluid after GHB dosing. As application of this assay, we showed that after a single subcutaneous dose of GBL at 0.67 g/kg, GHB concentrations at the regain of righting reflex were approximately 4.66 ± 1.15 mM in plasma ($n = 4$), 1.3 ± 0.11 μmol/g of whole brain homogenates ($n = 4$), and 80.4 and 81.0 μM in two cerebrospinal fluid samples.

4. Conclusion

We believe that the LC–MS procedure described in this report offers a sensitive means for determining GHB content in biological samples. The assay employs a deuterated internal standard, and its precision is therefore superior when compared to the HPLC assay reported by de Vriendt et al. [6] who could not find a suitable internal standard. The method has been proven sufficiently sensitive to detect endogenous GHB levels in various rat brain regions, and therefore may be employed for pharmacological studies in animals.

Acknowledgements

We thank Donna Ruszaj and David Soda for their excellent technical assistance, and Doanh Tran, Tony Dolan and Kathleen Boje for helpful discussions. This work was supported in part by NIH grant DA14988.

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