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Development and validation of a high-performance liquid chromatographic method for the determination of γ-hydroxybutyric acid in rat plasma

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Abstract

A method for the determination of γ-hydroxybutyric acid (GHB) in rat plasma was developed using solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) with UV detection. GHB was isolated from plasma using strong anion-exchange SPE columns. The chromatographic separation was performed on a C_{18} Aqua column. The lower limit of quantification was 10 μg/ml using 60 μl of plasma. The linearity of the calibration curves was satisfactory as indicated by correlation coefficients of >0.990. The within-day and between-day precision were <10% (n=24), the accuracy was nearly 101%. Plasma concentrations in rats after GHB infusion determined by HPLC–UV were compared with the corresponding concentrations determined with a validated gas chromatographic–mass spectrometric method by orthogonal distance regression. A good correlation was observed and a t-test indicated no significant differences from 0 and 1 for the intercept and slope, respectively.

Keywords: γ-Hydroxybutyric acid

1. Introduction

γ-Hydroxybutyric acid (GHB) is a naturally occurring substance produced by metabolism of the neurotransmitter γ-aminobutyric acid (GABA) [1]. GHB has been used as an intravenous anesthetic agent [2]. It is widely used as a drug of abuse.

The drug is almost completely eliminated by biotransformation [3]. The elimination after intravenous and oral administration is capacity limited in different species, which can be described by Michaelis–Menten kinetics [4,5].

Our laboratory is actually involved in a study of the influence of hemodynamic shock on the phar-

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macokinetics and the anesthetic effects of GHB in the rat.

Various gas chromatography (GC) and GC–mass spectrometry (MS) methods have been developed for measuring GHB in biological media [4–13]. High-performance liquid chromatography (HPLC) methods for the determination of GHB in biological samples are scarce, since GHB is a small polar molecule and absorbs light at a very low wavelength. In one HPLC method, GHB is determined in plasma after liquid–liquid extraction and fluorescence detection after dansyl derivatisation [14].

We have developed a HPLC method with UV detection at low wavelength for the separation and quantification of GHB in rat plasma using a C_{18} column endcapped with a hydrophilic polar reagent retaining both polar and non-polar compounds significantly longer than conventionally endcapped C_{18} columns. A solid-phase extraction (SPE) method that permits the direct isolation of GHB from the plasma samples is used.

2. Experimental

2.1. Chemicals and reagents

γ-Hydroxybutyric acid sodium salt (GHB), 3-hydroxybutyric acid, 2-hydroxybutyric acid, propionic acid, butyric acid, valeric acid, isobutyric acid, γ-aminobutyric acid, succinic acid and γ-butyrolactone were purchased from Sigma (St. Louis, MO, USA). Deuterated GHB (GHB-D6) (4-OH-2,2,3,3,4,4,-hexadeuterobutyric acid) was purchased from Radian (Austin, TX, USA). HPLC-grade acetonitrile and methanol, and the reagents 2-hydroxyvaleric acid, γ-valerolactone, δ-valerolactone were purchased by Acros Organics (Geel, Belgium). Analytical-grade formic acid, acetic acid and potassium dihydrogenphosphate were obtained from Merck (Darmstadt, Germany). Natrium heparine was obtained from B. Braun (Melsungen, Germany). Bond Elut cartridges, 1 ml capacity, packed with 100 mg of strong anion-exchange (SAX) material were used as supplied by Varian (Palo Alto, CA, USA). Water was purified with a Simplicity 185 ultra pure water system from Millipore (Bedford, MA, USA).

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a Varian 9010 solvent delivery system, an automatic injector, Merck–Hitachi AS 2000A, with a 100-μl loop (Merck), a Waters UV detector 4786 (Waters, Milford, MA, USA) and a HP 3395 integrator (Hewlett-Packard, Avondale, PA, USA). The compounds were separated on a C_{18}, Aqua column (150×4.6 mm, 5 μm) with a guard column (4.0×3.0 mm) packed with reversed-phase C_{18} material (Phenomenex, Torrance, CA, USA) at ambient temperature. The mobile phase consisted of 100% potassium dihydrogenphosphate solution (20 m*M*) at a flow-rate of 0.9 ml/min. UV detection was performed at 220 nm.

2.3. Solid-phase extraction procedure

A 60-μl volume of plasma was diluted with 60 μl of water, and 100 μl of the diluted sample was applied to a SAX cartridge, previously conditioned with 1 ml of methanol, 6 ml of formic acid (0.5 *M*) and 1 ml of water at a flow-rate of 1 ml/min. The cartridge was washed with 0.5 ml of water, 0.5 ml of water–methanol (1:1, v/v) and finally with 0.3 ml of methanol. To remove all residual liquid, a strong vacuum was applied for 10 min. GHB was eluted from the cartridges using 600 μl of acetonitrile containing 6% acetic acid. All solvents passed the cartridges at a flow-rate of approximately 1 ml/min. The eluate was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 150 μl of mobile phase. A volume of 100 μl was injected onto the HPLC column.

2.4. Calibration curve and validation of the method

A stock solution of GHB (100 mg/ml free acid equivalents) was prepared in water, dilutions were made in water, aliquoted and stored at −20°C. The calibration curve was constructed daily by adding 60 μl of each GHB-diluted standard solution to 60 μl of blank rat plasma. A volume of 100 μl was applied to the SPE cartridge. The calibration curve consisted of seven points ranging from 10 to 750 μg/ml GHB. Quality control (QC) samples at low (20 μg/ml), medium (300 μg/ml) and high (700 μg/ml) con-

centrations were prepared by spiking blank rat plasma with GHB-diluted standard solution. These samples were aliquoted and stored at −20°C until use. The calibration curve equations were estimated for the concentration range used by a linear least-squares regression model using a weighting factor $1/C^2$. Absolute recoveries of GHB from plasma were calculated by comparing the peak heights of extracted rat plasma spiked with GHB at concentrations of 20, 300 and 700 μg/ml, with those obtained from a standard solution of GHB dissolved in 150 μl of mobile phase. The selectivity of the assay was determined by analysis of blank plasma from 10 different rats. Accuracy, between-day and within-day precision of the method were determined by assaying six replicates of each of the three quality control samples on 4 different days. The accuracy was calculated at each test concentration and was obtained by dividing the mean measured concentration, obtained from all quality control samples on the 4 days, by the nominal concentration and multiplying with 100%. The within- and between-day precision were obtained by analyses of variance (ANOVA) for each test concentration. Accuracy and precision are expressed as analytical recovery and relative standard deviation (RSD), respectively. The limit of quantification (LOQ) is defined as the lowest concentration of the calibration curve, six times analyzed, for which the RSD is less than 20% and the accuracy is between 80 and 120%.

2.5. GC–MS analyses of GHB

GHB was assayed in rat plasma by a validated GC–MS method as described previously [10,15]. Plasma (20 μl) and 1 μg of internal standard (I.S.) (GHB-D6) were mixed with 45 μl of acetonitrile. The sample was centrifuged and the supernatant evaporated. GHB was derivatized at 90°C for 15 min after adding 75 μl of *N*,*O*-bis(trismethylsilyl)trifluoroacetamide to the tube. A volume of 1 μl of the solution was injected into the GC–MS system. The system was in the selected ion monitoring (SIM) mode (HP 5890 GC system and HP 5970 MS detector; Hewlett-Packard) using an Optima 1 column, 12 m×0.2 mm, 0.35 μm film thickness (Macherey Nagel, Düren, Germany) with the following temperature program: 50°C for 0.6 min, then increasing temperature at 10°C/min to 100°C and further at 50°C/min to 250°C, hold for 1 min (total duration: 9.6 min). Ions 233 and 239 were used as quantitative ions for GHB and GHB-D6, respectively. The calibration curve ranged from 10 to 200 μg/ml GHB. QC samples at low (20 μg/ml), medium (50 μg/ml) and high (200 μg/ml) concentration were analyzed in duplicate together with the samples. The quality control samples did not deviate more than 15% from the nominal value during the two runs performed. The LOQ is 2.5 μg/ml using 20 μl of plasma [15].

2.6. Comparison of samples analyzed with the HPLC–UV and GC–MS methods

The HPLC–UV and GC–MS methods were applied to the assay of GHB in rat plasma obtained from four male Wistar rats (415–520 g) after intravenous infusion of GHB dissolved in water (300 mg/ml, as salt). Each rat received a different dose: 1.6, 1.7, 4.9 and 5.1 g/kg/h with infusion times of 20, 25, 11 and 11 min, respectively. Arterial blood samples of 200 μl or 300 μl were taken from heparinized rats (500 U) as a function of time (12 time points) after the end of the infusion. Sampled blood was centrifuged and the plasma divided in equal parts and stored at −20°C. For both methods, samples not within the range of the standard curve were diluted with blank plasma. Rat plasma concentrations of GHB determined by HPLC–UV were compared with those determined using GC–MS by orthogonal distance regression [16]. The slope and intercept obtained were examined by a *t*-test to determine whether they were significantly different from 1 and 0, respectively. 95% confidence limits were calculated to check whether the ideal slope and intercept fell within these limits. The confidence limits were calculated as follows: [slope or intercept]±[standard deviation×t (α=0.05, n−2 degrees of freedom)] [17].

3. Results and discussion

3.1. Chromatography

Fig. 1 shows chromatograms of blank rat plasma, blank rat plasma spiked with 100 μg/ml of GHB,

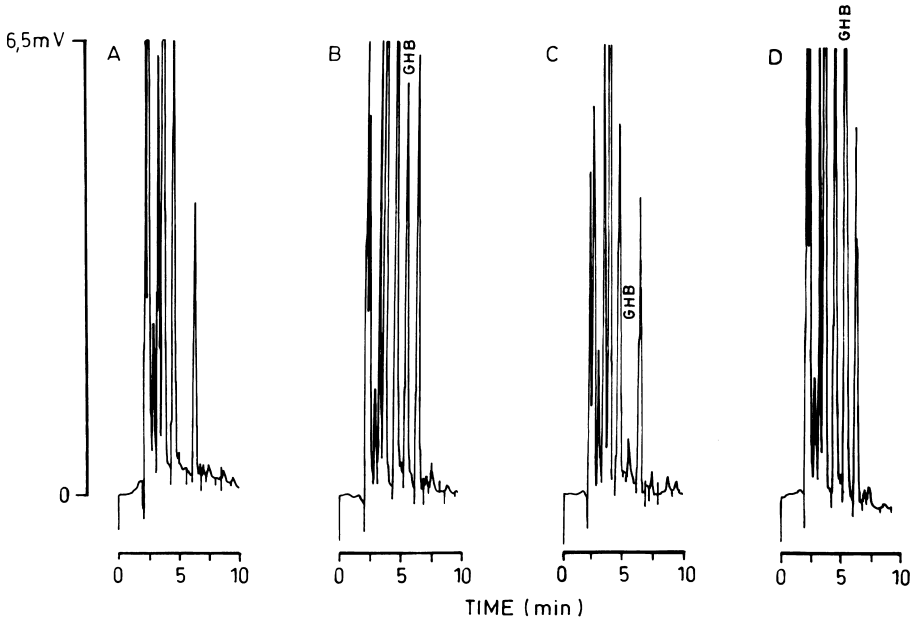

Fig. 1. Chromatograms of (A) blank rat plasma, (B) blank plasma spiked with 100 μg/ml GHB, (C) blank plasma spiked with 10 μg/ml GHB (LOQ) and (D) a rat plasma sample containing 390 μg/ml GHB after GHB intravenous infusion.

blank rat plasma spiked with 10 μg/ml and plasma obtained from a rat treated with GHB. GHB had a retention time of 5.6 min under the chromatographic conditions described. Peaks from endogenous compounds of the plasma are found before and after the GHB peak. These peaks are reproducible and do not interfere with the GHB peak. Several structural analogues to GHB were tested as possible I.S.s, but none of them were suitable (Table 1).

3.2. Validation

The mean absolute recovery at high, medium and low concentrations of GHB from plasma was 79±5.6% (n=18), independent of the GHB concentrations. The calibration curves, constructed in the 10–750 μg/ml GHB range, gave a mean correlation coefficient and slope of 0.993±0.003 and 475±21, respectively (n=4). Table 2 shows the mean back

Table 1
Retention times and reason for rejection of different compounds tested as internal standards

Name	Retention time (min)	Reason for rejection
γ-Aminobutyric acid	2.2	Interference
γ-Butyrolactone	6.0	Degradation
Succinic acid	6.3	Interference
3-Hydroxybutyric acid	7.3	Interference
2-Hydroxybutyric acid	7.8	Interference
Propionic acid	10.3	Evaporation
δ-Valerolactone	11.7	Degradation
γ-Valerolactone	16.9	Degradation
2-Hydroxyvaleric acid	24.0	Retention time too late
Butyric acid	32.5	Evaporation – retention time too late
Isobutyric acid	33.0	Retention time too late
Valeric acid	>70.0	Evaporation – retention time too late

Table 2
Mean back calculated concentrations of the calibration standards (n=4)

Concentration GHB added (μg/ml)	Mean calculated concentration±SD (μg/ml)
10	10±1
20	21±3
50	49±1
100	105±8
200	187±19
300	318±19
500	511±54
750	750±29

calculated concentrations of the calibration standards (n=4). These values ranged between 93 and 107% of the nominal value. None of the blank plasma samples collected from 10 different rats showed interference with GHB. The RSDs obtained in the study of within- and between-day precision were less than 10%. The accuracy was between 101 and 102% (Table 3). The LOQ was 10 μg/ml, using 60 μl of plasma, with a RSD of 10.6% and an accuracy of 86%. The precision and accuracy data demonstrate that the method is acceptable. The QCs stored at −20°C, were used for a period of 6 months and found to be stable.

3.3. Comparison of the HPLC–UV method with the GC–MS method

A comparison of the GHB plasma concentrations determined in paired samples from four rats using GC–MS and HPLC–UV methods is illustrated in Fig. 2. Although the precision of the two methods is in the same range, we decided to place the concentrations determined by GC–MS on the x-axis as this is the reference method. The slope and intercept of the orthogonal distance regression line with their

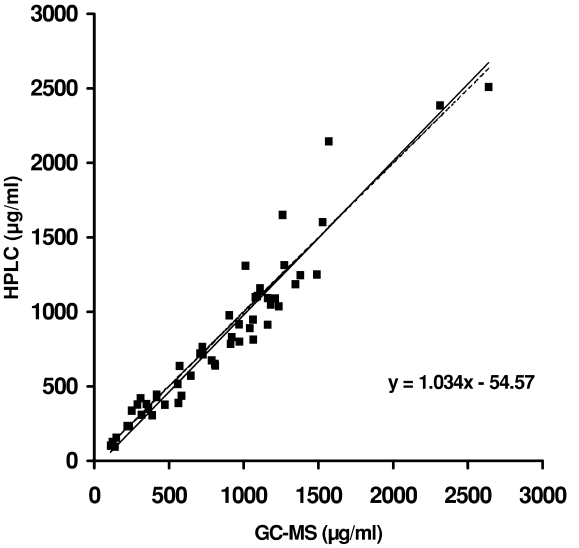

Fig. 2. Correlation between GHB plasma concentrations from four rats determined by HPLC–UV and GC–MS. (—) Line fitted by orthogonal distance regression, (- - -) line of equality.

confidence limits (α=0.05) were 1.034±0.081 and −54.6±80.4 μg/ml, respectively (n=53). The confidence intervals encompassed the ideal slope of unity and the ideal intercept of zero, indicating that the two methods are comparable to each other. Fig. 3 shows the plasma concentration–time curve in a rat after intravenous infusion of GHB at a rate of 5.1 g/kg/h for 11 min, using both HPLC–UV and GC–MS methods. The plasma concentration–time profiles were superimposable. The areas under the plasma concentration–time curve (AUCs), constructed with the trapezoidal rule from time 0 to the last sampling point, were calculated for each individual rat from the data using both methods. The average ratio of the AUCs obtained after HPLC–UV and GC–MS analysis of the plasma samples from each rat was 1.03±0.15 (SD) (n=4). This figure

Table 3
Precision (RSD) and accuracy (analytical recovery) of HPLC analysis of GHB in rat plasma

Concentration GHB (μg/ml)	Accuracy (%)	Within-day precision (%) (n=24, df=20)	Between-day precision (%) (n=4, df=3)
20	101.5	10.0	4.5
300	101.4	5.8	2.3
700	101.2	5.1	4.6

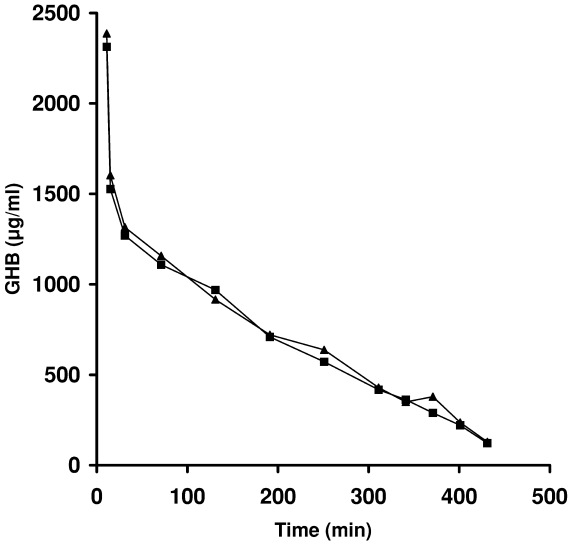

Fig. 3. Plasma concentration–time curve of GHB in plasma of a rat infused with GHB at a rate of 5.1 g/kg/h for 11 min, measured with GC–MS (■) and HPLC–UV (▲).

indicates that no important differences in plasma concentrations and AUCs are seen between the two assay methods.

4. Conclusion

We have developed and validated a new HPLC method for the determination of GHB in rat plasma. The method proves to be simple, specific, accurate and sensitive for the determination of GHB in a small volume (60 μl) of rat plasma. The overall low cost associated with liquid chromatography makes it an attractive alternative to GC–MS for the determination of GHB in rat plasma for pharmacokinetic purposes in laboratories where GC–MS is not available.

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