Validation of a simple gas chromatographic–mass spectrometric method for the determination of gamma-butyrolactone in human plasma

Yousuke Fukui[a],*, Eiji Matsusima[a], Kouichi Muramoto[a], Nobutaka Nagai[b], Koso Ohama[b], Kazumasa Yamashita[a]

[a]*Pharmacokinetics Research Laboratory, Taiho Pharmaceutical Co. Ltd., 224-2, Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-0194, Japan*
[b]*Department of Obstetrics and Gynecology, Faculty of Medicine, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan*

Abstract

A gas chromatographic–mass spectrometric (GC–MS) method is described for the determination of human plasma levels of gamma-butyrolactone (GBL) is described. The method is sensitive and simple. The plasma sample spiked with the internal standard was extracted by dichloromethane (CH_2Cl_2) in acidic conditions, and the concentrated organic layer was injected into GC–MS. Because of endogenous GBL in human plasma, the method used a standard calibration curve. The calibration curve was linear from 10 to 1000 ng/ml. The method has been validated for accuracy and precision with the relative error and C.V. for intra- and inter-day within 10%. GBL-spiked plasma samples stored at −80 °C were stable for a 3-month period. The stability of plasma samples after three cycles of freezing and thawing and of prepared samples on an autosampler for 48 h were demonstrated. Plasma concentrations of GBL before and after administration of UFT were 24.3±14.2 and 84.9±22.4 ng/ml, respectively.
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1. Introduction

Gamma-butyrolactone (GBL) or its acid form gamma-hydroxybutyric acid (GHB) are known as anesthetics [1], and it was discovered in mammalian brain [2]. Recently, GBL and GHB were investigated for activity against angiogenesis induced by tumor cells in the dorsal air sac (DAS) with five cell lines (murine renal carcinoma RENCA, human gastric cancer 4-1 ST, human small-cell lung carcinoma LX-1 and human colon carcinoma DLD-1 and KM-20C). Basaki et al. [3] used the anticancer drugs tegafur (FT), UFT (assorted drug of FT and uracil, molar ratio 1:4), 5-fluorouracil (FUra) and doxifluridine (5′-DFUR), and reported that UFT demonstrated significant anti-angiogenic activity in a dose-dependent manner, while FUra and 5′-DFUR were less effective. Also, FT, GHB and GBL inhibited angiogenesis induced RENCA cells and the

*Corresponding author. Fax: +81-886-665-6206.
E-mail address: y-fukui@taiho.co.jp (Y. Fukui).

inhibitory effect was increased with administration by continuous infusion. Another report demonstrated that GHB inhibits the angiogenesis induced by intact human cancer cell lines and that mediated by vascular endothelial growth factor (VEGF) [4]. These studies suggested that GBL and GHB are involved in expression of the anti-angiogenic activity of UFT and that they have suitable pharmacokinetic profiles. Au and Wolfgang demonstrated that FT is considered as a metabolic prodrug, releasing FUra and 4-hydroxybutane which can be further oxidized to GHB and hence GBL. They found that the plasma concentration of GBL after administration of 2000 mg/ m^2 of FT was about 20 μM by the GC-flame-ionization method [5]. Fieler et al. reported 27 mg/ ml of GBL in a patient with fatal poisoning [6]. Ferrara et al. reported 80–100 μg/ml of human plasma concentration of GBL. In this method, GBL was extracted with benzene and it was analysed by GC–MS [7]. These studies were monitored after administration of GBL or GHB, and the lower limit of quantification was insufficient for our analysis. With the therapeutic dose of UFT (approximately 300–600 mg/body per day), the plasma concentration of GBL was inferred to be about 100–200 ng/ml.

The analysis method of GBL demonstrated by Au and Wolfgang was modified. In the original method, GBL was extracted from the diluted plasma samples by chloroform after being lactonized by concentrated sulfuric acid. The extracted chloroform layer was evaporated to about 50 μl, and then it was injected into the GC [5]. The range of this method of analysis was 200–200 000 ng/ml. Other studies used GC–MS to determine GBL or GHB, but their lower limits of quantification were 200–5000 ng/ml [7–9]. Moreover there is no study preparing QC samples for validation. To examine the pharmacokinetics of GBL or GHB, for anti-angiogenic activity, after oral administration of UFT, a more sensitive analytical method is needed.

The aim of this study was to develop a sensitive and accurate analytical method to detect plasma GBL levels after therapeutic oral administration of UFT. Because GBL is a volatile solvent, the GC–MS method is suitable to assay it. In order to improve sensitivity, negative ion chemical ionization (NICI) MS was applied. The pretreatment of plasma sample

described in this study is simple, only extraction is required and no derivatization. The analysis method described here prepared QC samples, and then the method was validated.

2. Materials and methods

2.1. Chemicals

GBL was purchased from Wako (Osaka, Japan) and GBL-d_6 from Aldrich (Milwaukee, USA). HPLC-grade methanol (MeOH), dichloromethane (CH_2Cl_2) and 6 M hydrochloric acid were obtained from Wako. Water was purified by Milli-Q SP-TOC (Nihon Millipore, Kogyo, Japan).

2.2. Blood samples and healthy subjects

Blood samples were obtained from four uterine cancer patients and five healthy volunteers. All subjects gave written informed consent. The heparinized blood was centrifuged to separate erythrocytes from plasma. The patients had given 200 mg×3/ body per day of UFT for 5 days. Blood samples were taken before administration and on the final day of administration of UFT. The collected time of blood on the final day was 2–2.2 h after administration of 200 mg of UFT. After removing the plasma, it was stored at −80 °C until sample preparation.

All of the healthy volunteers had no acute illness or revealed clinical signs. They received no medication for at least a week.

2.3. Instruments

GC–MS was carried out using a Trace-GC gas chromatograph and AS2000 automatic sampler interfaced with a Trace-MS quadrupole mass spectrometer (all from ThermoQuest, San Jose, CA, USA). NICI was carried out with isobutane as the reagent gas. Ionization was initiated at 70 eV with an emission current of 150 μA. The source temperature was 210 °C, and the GC interface temperature was 250 °C. The GC column was interfaced directly to the ion source. Gas chromatographic separation was carried out on a DB-WAX capillary column (30 m×0.32 mm I.D., film thickness 0.25 μm, J & W

Scientific, Folsom, CA, USA). The column temperature was programmed with a two-ramp temperature program: the oven was heated at 50 °C for 1 min, then increased by 20 °C/min to 190 °C for the first ramp and by 40 °C/min to 250 °C for the second ramp, and then the oven temperature was held for 2 min. Xcalibur (version 1.2) was used for instrument control and data acquisition.

2.4. Sample preparation and GC–MS analysis

2.4.1. Preparation of standard solutions and QC solutions

A standard stock solution of 400 μg/ml of GBL, an internal standard stock solution of 400 μg/ml of GBL-d_6 and a QC stock solution of 800 μg/ml of GBL were prepared in methanol. Calibration standard solutions of 4000, 1000, 200, 100 and 40 ng/ml were prepared by diluting a standard stock solution with water. An internal standard solution of 2000 ng/ml was prepared by diluting the internal standard stock solution with methanol. QC solutions of 8000, 2000 and 200 ng/ml of GBL were prepared by diluting QC stock solution with water. All solutions were stored at 4 °C.

2.4.2. Preparation of calibration standards, matrix-added standards and blank samples

A 0.05-ml portion of standard solution and 0.05 ml of internal standard solution were added to 0.15 ml of water or blank plasma to prepare calibration standards or matrix-added standards. A 0.25-ml portion of water was used as a double blank sample. A 0.05-ml portion of internal standard solution was added to 0.2 ml of water to prepare a single blank sample.

2.4.3. Preparation of QC samples and diluting assay sample

QC solutions were diluted 10 times by blank plasma to prepare the QC samples (QC-H: 800 ng/ml, QC-M: 200 ng/ml and QC-L: 20 ng/ml). The standard stock solution was diluted eight times with water, and then the solution was diluted 10 times with blank plasma to prepare the diluting assay sample. Before sample pretreatment, the diluting assay sample was diluted 10 times by water. For the repeatability study, QC-H, QC-M and QC-L were

used. For the stability study, QC-H and QC-M were used. QC samples and diluting assay sample were stored at −80 °C until analysis.

2.4.4. Sample pretreatment and GC–MS analysis

A 0.5-ml portion of 6 *M* hydrochloric acid and 0.05 ml of internal standard solution (2 μg/ml GBL-d_6) were added to 0.2 ml of plasma samples. After adding 2 ml of CH_2Cl_2, the mixture was shaken for 10 min, and then centrifuged at 1900 *g* for 15 min at 5 °C. The organic layer was transferred to another test-tube by glass pipette. CH_2Cl_2 extraction was repeated once. The combined organic layer was evaporated to about 100 μl at 35 °C under a gentle stream of nitrogen. The concentrated organic layer was transferred to an injection vial and an aliquot (1 μl) was injected into the GC–MS system. GBL and internal standard (GBL-d_6) were detected by a selected ion monitoring procedure at $m/z = 85$ and $m/z = 90$, respectively.

2.4.5. Calibration and calculations

Calibration standards were prepared using concentrations of 1000, 250, 50, 25 and 10 ng/ml. The peak area of GBL to the internal standard was used as the assay parameter. The peak area ratios were plotted against theoretical concentrations. Calibration curves were obtained from weighted ($1/x^2$) least-squares linear regression analysis of the data.

2.5. Validation

The linearity of calibration was tested ($n = 5$). A 200-μl portion of QC sample of each concentration was extracted and then assayed, and calculated against the calibration curve. Precision was calculated at concentrations of 800 (QC-H), 200 (QC-M) and 20 (QC-L) ng/ml of QC samples by the interpretation of the intra-day variations (repeatability) and inter-day variations (reproducibility) five times. Repeatability was determined by comparison of the concentrations of the same QC samples extracted and injected during the same day. To test reproducibility, the same QC samples, were extracted and injected on five different days. Precision around the mean value should be within 15% sample concentration of error of the theoretical concentration and coefficient of variation (C.V.). Dilutional analysis

was carried out at a concentration of 5000 ng/ml, five times. The concentration of the diluted sample was calculated 10 times. Stability on an auto sampler was tested with the same prepared sample, once immediately after preparation and again 48 h after preparation. Freeze-thaw stability was tested by analysis of QC-H and QC-M samples, after three cycles of freezing and thawing. The samples were frozen at $-30\,^\circ$C and thawed in water. Stability in plasma was tested by assaying the frozen QC-H sample and QC-M sample after storage for 1 month and 3 months.

Calculation of sample concentration was carried out against a standard calibration curve. Precision around the mean value should be within 15% sample concentration of error of the theoretical concentration and coefficient of variation (C.V.). Since endogenous GBL peak was detected for blank plasma, the theoretical concentration was calculated by the sum of added GBL concentration and the concentration of the blank sample.

3. Results and discussion

3.1. Analytical method

The analytical method described here modified the method of Au and Wolfgang [5]. To improve sensitivity, NICI-MS was applied. The peak detection was about 10 times more sensitive, compared with positive ion electron impact ionization. Moreover, there are no background peaks near GBL or the internal standard peak (Fig. 1). The original method used chloroform solution of GBL to prepare calibration standard solutions, and no QC samples were prepared. To prepare QC samples, this method used methanol stock QC solution, and then it was diluted with water. The original method used chloroform to extract GBL from plasma samples, and used concentrated sulfuric acid to lactonize GHB. This method used 6 M hydrochloric acid to lactonize GHB, because concentrated sulfuric acid is not easy to handle. GHB and delta-valerolactone (DVL: original method used as an internal standard) are volatile, so careful evaporation of the extracted organic layer was necessary. To avoid the influence of different volatilities of GBL and DVL, this method used GBL-d_6 as an internal standard. This method has

satisfactory sensitivity, and the final concentration volume of the organic layer was up to 50–100 μl.

3.2. Linearity of calibration curve and endogenous GBL in human plasma

The chromatograms of QC-H sample and blank plasma are shown in Fig. 1. In GC–NICI-MS analysis, the major fragment ion was monitored. The observed retention time was 5.89 min for GBL and GBL-d_6 (Fig. 1A,B). There was a GBL peak (retention time 5.89 min) in the chromatogram for blank plasma (Fig. 1C). The peak on blank plasma chromatogram indicated endogenous GBL. The blank plasma concentration of GBL reported by Gibson et al. from six children (ages 1 month to 12 years) and one adult was about 100 ng/ml [10]. In this study, the plasma concentration of endogenous GBL was assayed by quantifying five blank samples spiked with internal standard and the mean concentration of blank samples was calculated (Table 1). The sum of the mean plasma concentration of endogenous GBL (12.2 ng/ml) and added GBL concentration was used as a theoretical concentration for QC samples.

The calibration curve for standard and matrix-added standard was linear from 10 to 1000 ng/ml, with r values of 0.999 and 0.996, respectively (Table 2). The slope and intercept of the standard calibration curve were 0.00150 and 0.00274, respectively, while for the matrix-added standard calibration curve they were 0.00147 and 0.0206, respectively. The standard calibration curve was parallel to the matrix-added standard calibration curve. The ratio of the intercept of the matrix-added calibration curve, was indicated 11.9 ng/ml of recalculation used by the standard calibration curve. This value was very close to the blank plasma concentration of GBL (12.2$\pm$0.379 ng/ml). The difference between the intercepts for the standard and matrix-added standard indicates the endogenous plasma GBL added to the standard calibration curve. Since this method uses the standard calibration curve, the quantified plasma sample concentration of GBL includes the endogenous GBL concentration.

3.3. Precision, accuracy and diluting assay

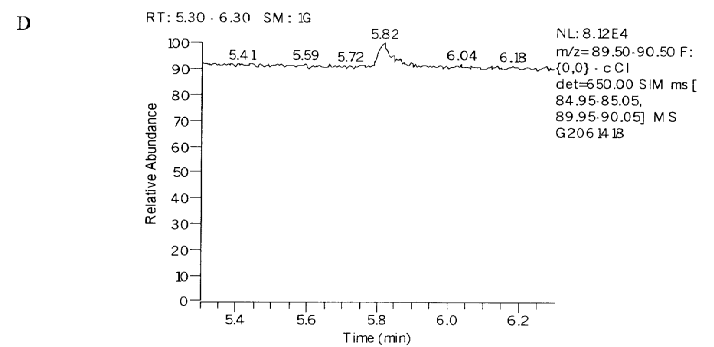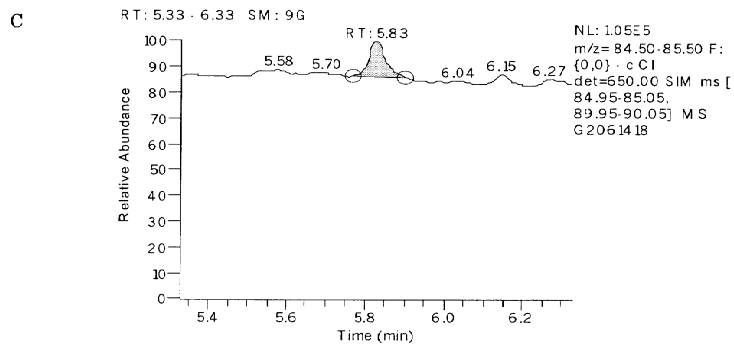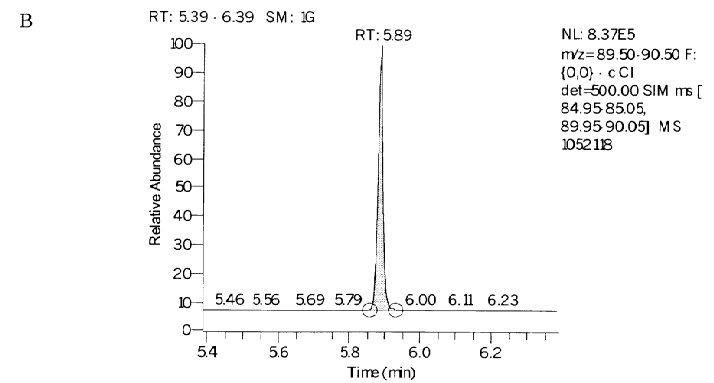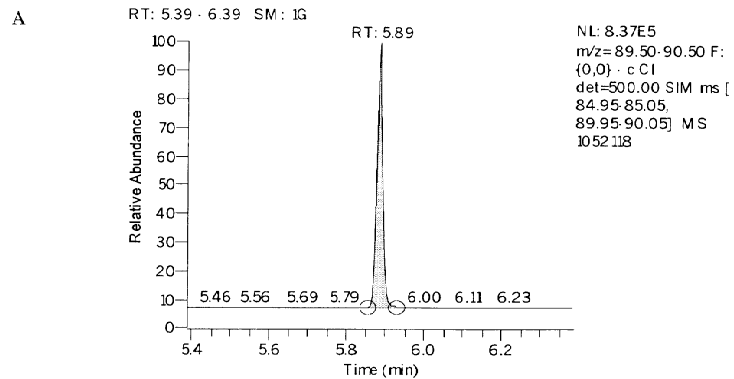Intra- and inter-day repeatability in human plasma was assayed by spiked internal standard the extracted

Fig. 1. Chromatogram of QC-H sample and blank plasma. (A) GBL ($m/z = 85$) of QC-H sample. (B) Internal standard ($m/z = 90$) of QC-H sample. (C) GBL of blank plasma. (D) Internal standard of blank plasma.

Table 1
The concentration of GBL in blank plasma

Healthy volunteer	Plasma concentration of endogenous GBL (ng/ml)	Mean±SD
1	12.5	
2	11.5	12.2
3	12.3	±
4	12.3	0.379
5	12.1	

three concentrations of QC samples (800, 200 and 20 ng/ml), and calculated against a calibration curve. The intra-day repeatability was determined by extracted plasma samples in replicates the same day. The inter-day repeatability was repeated for different days with the same QC samples. The accuracy, expressed as percent deviation of the observed concentration from the theoretical concentration was evaluated along the relative error. The results of accuracy data, intra- and inter-day repeatability are shown in Table 3. The relative error of intra- and inter-day repeatability was less than ±6%. The coefficient of variation (C.V.) of the intra- and inter-day repeatability was less than ±8%. This method

thus exhibits sufficient repeatability and reproducibility.

The diluting assay was confirmed to determine concentrations over the range of calibration. Calibration samples prepared over the highest range ($n = 5$) were diluted 10 times volume with water. Then they were pretreated in the same way as for QC samples and assayed. The relative error and C.V. of the diluting assay were 1.2 and 2.3%, respectively. For the diluting assay, theoretical concentration was used, not addition of blank plasma concentration of GBL, because blank matrix was diluted at this assay. Samples over the upper limit of quantification can be diluted with water, before extraction (Table 3).

3.4. Stability

The stability of GBL in plasma samples was studied. A GBL-spiked plasma sample was placed in polypropylene tubes in storage at $-80\,^{\circ}C$ for 1- and 3-month periods. The concentration of storage sample was quantified. GBL concentration after storage and assayed concentration/theoretical concentration values are shown in Table 4. QC-H and QC-M sample assayed concentrations were within ±7%.

Table 2
Standard calibration curve and matrix added calibration curve

	Coefficient of the linear regression analysis (mean±SD)	Slope ($\times 10^{-3}$) (mean±SD)	Intercept ($\times 10^{-3}$) (mean±SD)
Standard calibration curve	0.999±0.000538	1.50±0.00973	2.74±0.927
Matrix added calibration curve	0.996±0.00314	1.47±0.0163	20.6±2.39

Table 3
The accuracy and precision of intra-day, inter-day and diluting assay for determination of GBL in human plasma

	QCs	Theoretical concentration (ng/ml)	Mean calculated concentration±SD (ng/ml)	C.V. (%)	Accuracy (%)
Intra-day	QC-H	812.2	764.1±5.77	0.8	−5.9
($n = 5$)	QC-M	212.2	226.5±5.39	2.4	6.7
	QC-L	32.2	29.78±1.51	5.1	−7.5
Inter-day	QC-H	812.2	825.6±47.6	5.8	1.6
($n = 5$)	QC-M	212.2	223.8±8.96	4.0	5.5
	QC-L	32.2	33.28±1.30	3.9	3.4
Diluting assay ($n = 5$)		5000	5117±62.0	1.2	2.3

Table 4
Stability of GBL in storage at $-80\,^{\circ}\text{C}$

	Theoretical concentration (ng/ml)	Preserved period (months)	Mean calculated concentration$\pm$SD (ng/ml)	Mean calculated concentration/ theoretical concentration (%)
QC-H	812.2	1	776.2$\pm$13.0	95.6
		3	821.6$\pm$5.67	101.2
QC-M	212.2	1	205.1$\pm$7.58	96.7
		3	217.3$\pm$0.856	102.4

The difference in initial concentration with assayed concentration was within $\pm10\%$. In addition GBL in plasma sample was stable for 3 months. Stability after three freeze and thaw cycles was tested using QC-H and QC-M samples. The concentration after freezing and thawing was within $\pm8\%$ (Table 5). Samples were thus stable on freezing and thawing. The stability on autosampler was studied. A prepared sample was placed on the autosampler for 48 h, and then the sample solution was injected into the GC–MS. The peak area ratio was compared with the peak area ratio for the sample immediately after treatment (Table 6). The area ratio was not changed for 48 h. Prepared samples were stable for the analytical period. Stock standard solutions, stock internal standard solution and prepared solutions stored at $4\,^{\circ}\text{C}$ were stable for a 3-month period.

3.5. Plasma concentration of GBL before and after oral administration of UFT

The plasma concentration of GBL is shown in Fig. 2. The mean concentration of UFT before administration was 24.3 ± 14.2 ng/ml and after oral administration, it was 84.9 ± 22.4 ng/ml. The plasma concentration of GBL has increased after oral administration of UFT. This increase in plasma level of GBL was found in all patients.

This change cannot be detected with usual analytical methods. A detailed pharmacokinetic study of GBL after oral administration of UFT using this method will be reported elsewhere. This method is useful for studying the pharmacokinetics and anti-angiogenic activity of GBL.

Table 5
Stability of QC samples after three cycles of freezing and thawing

	Theoretical concentration (ng/ml)	Mean calculated concentration$\pm$SD (ng/ml)	Mean calculated concentration/ theoretical concentration (%)
QC-H	812.2	855.5$\pm$3.34	105.3
QC-M	212.2	228.3$\pm$3.21	107.6

Table 6
Stability of QC samples in autosampler

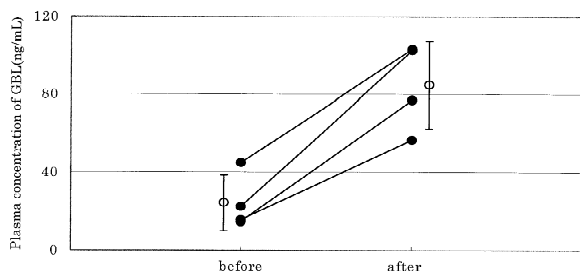|	Theoretical concentration (ng/ml)	Mean area ratio immediately after treatment$\pm$SD (ng/ml)	Mean area ratio left for 48 h on autosampler$\pm$SD (ng/ml)
QC-H	812.2	1.12$\pm$0.01	1.07$\pm$0.01
QC-M	212.2	0.315$\pm$0.02	0.306$\pm$0.01

Fig. 2. Variation of plasma concentration of GBL in four patients before and after oral administration of UFT. ●, GBL level of each patients; ○, mean±SD.

4. Conclusions

The analytical method developed in this study for the determination of human plasma levels of GBL used NICI is sensitive, accurate and precise and has a simple pretreatment. This study indicated that GBL is stable in plasma for 3 months, so plasma samples can be stored until analysis. This method can be applied to the pharmacokinetic analysis of GBL, after oral administration of a therapeutic dose of UFT.

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