Determination of pregabalin in human plasma by electrospray ionisation tandem mass spectroscopy

Gaurang R. Shah,
Chinmoy Ghosh[1], Bharat T. Thaker

Department of Chemistry,
V. N. S. G. University, Udhna Magdalla
Road, Surat - 395 007, Gujarat,
[1]Jiwaji University, Gwalior - 474 001,
M.P., India

ABSTRACT

A rapid, precise, specific, and accurate Electrospray Ionisation Tandem Mass Spectrometry (ESI-MS / MS) method has been developed and subsequently validated, for the determination of pregabalin (PB) in human plasma. Gabapentin (GB) was used as the internal standard. PB and GB were extracted from the plasma using a combination of deproteinization, using 0.1% formic acid and liquid–liquid extraction, using methylene chloride. PB and GB were separated using the Hypurity advance column (50 mm × 4.6 mm, 5 μm) and mobile phase, consisting of methanol : 0.1% formic acid (80:20 v / v). PB was determined by using ESI-MS / MS in positive ion mode, with the help of the API 2000 spectrophotometer, operated in a multiple reaction monitoring mode. The parent-to-product ion combination of m / z 160.2→55.1 and 172.2→95.0 was used to quantify PB and GB, respectively. The assay was validated in the concentration range of 99.79 – 4019.90 ng / mL for PB. The limit of quantification (LOQ) was identifiable and reproducible at 99.79 ng / mL. The method has been successfully applied to study the pharmacokinetics of PB in healthy male volunteers.

Key words: Bioanalysis, ESI-MS/MS, gabapentin, pharmacokinetics, pregabalin

INTRODUCTION

Pregabalin (PB), [(S)-3-aminomethyl-5-methyl hexanoic acid] as shown in Figure 1, is an alpha2-delta (α2δ) ligand for the management of fibromyalgia, which has analgesic, anxiolytic, and anticonvulsant activities. The α2δ ligand is an auxiliary protein associated with voltage-gated calcium channels, and PB binds potently to this subunit.[1] Avid binding at this sight reduces calcium influx at the nerve terminal, and therefore, reduces the release of several neurotransmitters, including glutamate, norepinephrine, and substance P.[2-6] This has recently been approved for the treatment of partial seizures in patients with epilepsy and for the treatment of neuropathic pain, in Europe.[7,8] Approaches for measuring PB in plasma / serum has been described by reversed phase high-performance liquid chromatography (RPHPLC),[9-11] but these methods that involve derivatization are also complex and critical. There are very tedious methods available for the pharmacokinetic study of PB in human plasma / serum using liquid chromatography-mass spectrometry (LC-MS / MS). Hence, it is necessary to develop a simple and sensitive method for the determination of PB in human plasma using ESI-MS / MS.

Address for correspondence

Mr. Gaurang Shah, Department of Chemistry,
V. N. S. G. University, Udhna Magdalla Road, Surat - 395 007,
Gujarat, India. E-mail: gshah1975@yahoo.co.in

The method was developed with different approaches,[12,13] which can be successfully applied to the study pharmacokinetics of PB in healthy male volunteers.

Experimental
Instrumentation
The HPLC-MS / MS system consists of an Agilent 1100 Binpump, Autosampler, and API 2000 (Applied Biosystem, MDS SCIEX) triple quadrupole tandem mass spectrometer, with the analyst software (Version 1.4.1 CA, USA).

MATERIALS AND METHODS

PB and GB was supplied by Macleods Pharmaceuticals Ltd. (India). Human plasma was procured from the Prathma Laboratory, Ahmedabad, India. All solvents used were of HPLC grade and supplied by J.T.Baker (Germany). All the other chemicals were of analytical grade. Ultra pure water
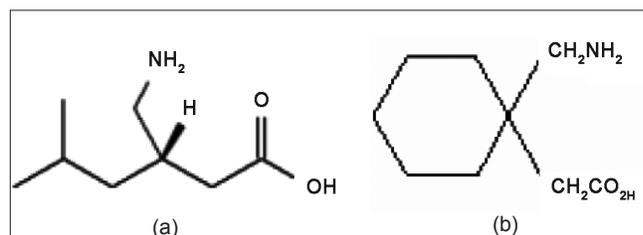
Figure 1: (a) Pregabline (PB) and (b) Gabapentin (GB)

was obtained from the Millipore Q-plus water purification system (Bedford, MA). PB capsule formulation was supplied by Macleods Pharmaceuticals Ltd. Chromatographic separation was achieved by using the Hypurity advanced HPLC column (50 mm × 4.6 mm, 5 μm), which was supplied by Thermo Scientific (U.K.).

Chromatography and Tandem Mass Spectrometry

The liquid chromatography system consists of the LC pump and autosampler from theAgilent 1100 series SPLC system (India). The detector was the API-2000 (Applied Biosystem / MDS Sciex, Toronto, Canada) mass spectrometer. Hypurity advance column (50 mm × 4.6 mm, 5 μm Thermo Electron Corporation, USA) was used as a stationary phase. The isocratic mobile phase consisting of Mehanol: 0.1% acetic acid (80:20, v / v) was used throughout the analysis. The flow rate of the mobile phase was 0.250 mL / minute with a splitter. The column oven temperature was kept at 40°C and the sample injection volume was 10 μL.

The Mass Spectrometer was operated in the multiple reaction monitoring (MRM) mode. The sample introduction and ionization technique was electrospray with positive polarity. The ion spray voltage was 4500 KV and the source temperature was 450°C. Nitrogen sheath gas (GAS1) and auxiliary gas (GAS2) were 25 psi and 30 psi, respectively. The mass parameter and multiple reaction monitoring (MRM) condition of each individual analyte is summarized in Table 1. The retention times of PB and GB were observed at 1.27 and 1.40 minutes, respectively, as shown in Figures 2a and b. Quantification was performed with the MRM of the transitions of m / z 160.2→55.1 for PB and m / z 172.2→95.0 for GB, with a scan time of 0.2 seconds per transition.

Preparation of Standard Solutions

Fresh stock solutions were prepared with the weighing of compounds. Primary stock solutions of concentration 1000 μg / mL of PB and GB were prepared in methanol.

The working stock solution of PB was diluted using 90% methanol in water to a suitable concentration 100.00, 200.00, 400.00, 800.00, 1800.00, 2600.00, 3200.00, or 4000.00 ng / mL to obtain the calibration curve range. The Quality control samples (QC) of low (LQC), medium (MQC), and high (HQC) concentrations, that is, 300.00 ng / mL, 1900.00 ng / mL, and 3000.00 ng / mL were also prepared.

The working stock solution of GB was diluted to a suitable concentration (50 μg / mL) in the mobile phase, for spiking. The stock solution and working solution were stored in a refrigerator, below 10°C.

Sample Preparation

In 400 μL of plasma, 50 μL of working calibration standard solution and quality control samples were added, respectively, along with 50 μL of Internal Standard working

solution (50 μg / mL) and vortexed for 15 seconds. Four milliliters of 0.1% formic acid in methylene chloride was added and allowed to be multi-vortexed at 100 RPM for 20 minutes. Next, the samples were centrifuged for 5 minutes at 4000 RPM and 4°C. The organic layer was separated and evaporated under a stream of nitrogen at 50°C. The residue was reconstituted with 200 μL of the mobile phase, vortexed for 30 seconds, and transferred to the pre-labeled HPLC vial.

Assay Validation

The method validation assays were carried out according to the currently accepted US Food and Drug Administrator (USFDA) Bioanalytical Method validation guidelines.[14]

The selectivity / specificity of the method was tested by screening six different batches of healthy human blank plasma. Each blank sample was tested for interference if any, using the proposed extraction procedure and chromatographic / spectroscopic conditions.

The matrix effect, that is, enhancement or suppression of the response of the drug / analyte due to the matrix compound was evaluated by comparing the analyte response in the extracted blank sample, wherein the analyte was externally spiked and then reconstituted to the final volume, with that of and non-extracted or aqueous sample. The effect of the same concentration was checked at low, middle, and high QC concentrations. As comparable response was observed, it was concluded that the matrix effect did not exist.

The linearity was tested for the ranges of concentration between 99.79 and 4019.90 ng / mL. Standard calibration curves of eight points (non-zero standards) were used. In addition, a blank plasma sample was also analyzed, to confirm the absence of interferences, although this sample was not used to construct the calibration function. The intra-day precision and accuracy of the assay was measured by extracting six replicates of PB at each QC level. The inter-day precision and accuracy was determined at each QC level on three different days. The acceptance criterion for precision and accuracy was that the deviation values had to be within 15% of the actual values. The absolute recovery of the extraction was determined by comparing the peak area obtained from the plasma sample, with the peak areas obtained by the direct injection of pure PB standard solution in the mobile phase at the QC level. The quantification was

Table 1: Intra- and inter-day precision and accuracy of the measurement of PB when used for positive ion detection

Concentration (ng/mL)	Intra-day		Inter-day	
	Precision	Accuracy	Precision	Accuracy
300.64	6.38	94.96	6.82	94.55
1902.79	4.38	92.72	5.22	93.38
3010.74	4.52	96.16	5.36	96.89

performed by applying peak area ratios of PB to the internal standard. To evaluate the different stabilities of the analyte in the plasma, six replicate samples, each of low and high QC concentration, were allowed to undergo the stability treatment and then analyzed against a fresh calibration curve and compared with the fresh QC samples.

Freeze thaw stability at below -50°C for four cycles, bench top stability of six hours at room temperature, and post-extraction stability in the autosampler were proved for 48 hours. Stabilities of the analyte and internal standard in the solution were also proved.

### Subject and Pharmacokinetics Study Protocol

The maximum plasma concentration (C_{max}) and the time of occurrence (T_{max}) of PB were obtained directly from the observed data. The area under the plasma concentration-time curve (AUC) from time zero to the last measured concentration (AUC_{0-t}) was calculated according to the linear trapezoidal rule.

RESULT AND DISCUSSION

Recovery, Linearity, Precision, and Accuracy

The Hypurity advance column and the mobile phase used for the determination gave a well-defined separation between the drug, internal standard, and endogenous components, as it could be confirmed from the blank and externally spiked chromatograms shown in Figure 3a and b. The retention time for PB and GB were observed at 1.27 minutes and 1.40 minutes, respectively.

The linear calibration curve was proven for PB in the range of 99.79 to 4019.90 ng / mL. The coefficient of correlation for all measured sequences was greater than 0.9900. The intra- and inter-day precision and accuracy are summarized in Table 1. Results demonstrated that the intra-day precision (% CV) ranged from 4.38 to 6.38% and inter-day precision ranged from 5.22 to 6.82%. The intra-day accuracy (% Nominal) ranged from 92.72 to 96.16% and the inter-day accuracy (% Nominal) ranged from 93.38 to 96.89%. The absolute recovery determined for PB was seen to be
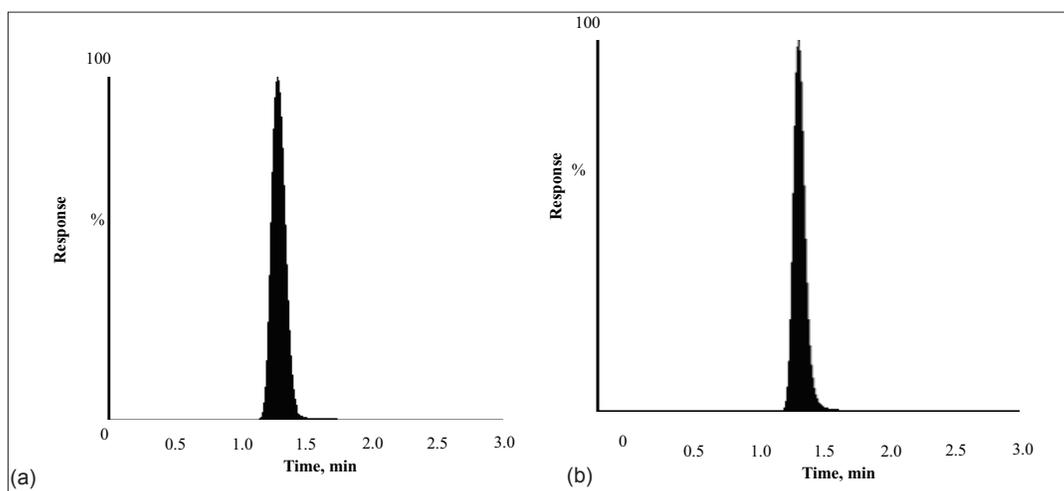
Figure 2: (a) Chromatogram of PB R.T – 1.27 (b) Chromatogram of GB R.T – 1.40
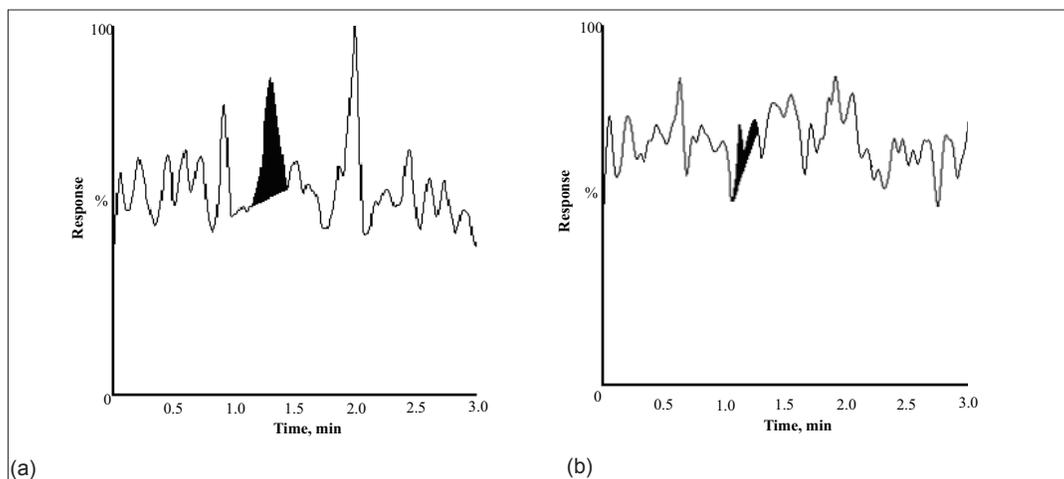
Figure 3: (a) Chromatogram of blank plasma of PB (b) Chromatogram of blank plasma of GB
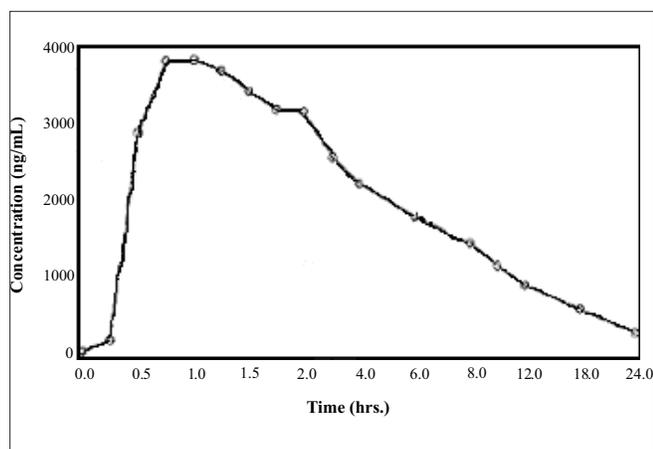
Figure 4: Plot of PB mean plasma concentration (ng / mL) versus Time (hours) (n = 23)

Table 2: Pharmacokinetic parameters of PB after oral administration of 150 mg of the dose

	Pregablin (Mean±standard deviation, n=4)
C_{max} (μg / mL)	4642.03±866.97
AUC_{0-t} (μg*hr / mL)	29449.86±3754.79
$AUC_{0-\infty}$ (μg*hr / mL)	31570.06±4479.05
T_{max}	1.0978±0.4631
K_{el}	0.1208±0.01597
$T_{1/2}$	5.83146±0.77781

consistent, precise, and reproducible. The mean recoveries of the three QC levels (LQC, MQC, and HQC levels) were 16.6, 18.7, and 19.0%, respectively.

Stability Studies
The result of the freeze-thaw stability indicated that the analyte was stable in plasma for four freeze-thaw cycles, when stored at -50°C and thawed to room temperature. Long-term stability indicated that storage of PB plasma samples at below -50°C was adequate when stored for 30 days and no stability-related problems would be expected during routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies.

Pharmacokinetic Study
The method was applied to determine the mean plasma concentration-time curve of the PB capsule in 23 healthy male volunteers [Figure 4]. After oral administration of 150 mg PB, the T_{max} and C_{max} values were found to be 1.0978 µL and 4642.03 µL, respectively. Plasma concentrations declined with $t_{1/2}$ of 5.83 hours [Table 2].

CONCLUSIONS

The proposed method of analysis provided a sensitive and specific assay for the determination of PB in human plasma. No significant interference caused by endogenous components was observed. A simple liquid–liquid extraction procedure and a short analysis time was important for large sample batches. It was seen that this method was suitable for the analysis of PB in plasma samples collected for pharmacokinetic, bioavailability or bioequivalence studies.

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