



A Novel UPLC–MS/MS Method for Determination of γ -Amino Butyric acid Analogue in Human Plasma: Application to Pharmacokinetic Study

Anju Aji^{*1,2}, Sarita Karthikeyan^{1,2}, Sarabjit Singh², Shivanand P. Puthli¹

¹ J.J.T University, Jhunjhunu, Rajasthan, India.

² Panacea Biotec Ltd, 72/3 TTC Industrial Area, Mahape, Navi Mumbai, Maharashtra 400710, India.

ARTICLE INFO

Article history:

Received on: 24/04/2013

Revised on: 16/05/2013

Accepted on: 05/05/2013

Available online: 27/06/2013

Key words:

γ -amino butyric acid

LC-MS/MS

UPLC

Human plasma

ABSTRACT

Pregabalin, a structural analogue of γ -amino butyric acid, is an anticonvulsant used for neuropathic pain. To facilitate its pharmacokinetic study in human subjects, ultra-performance liquid chromatography–mass spectrometric method employing positive electrospray ionization was developed for the determination of Pregabalin concentration in human plasma. Pregabalin together with the internal standard was extracted from 125 μ l of human plasma by solid phase extraction. The chromatography was performed using an amide column and multiple reaction monitoring detection mode was used for the quantification of Pregabalin in plasma. The validation of the method including sensitivity, linearity, reproducibility and stability was examined. The lower limit of quantification (LLOQ) of the developed method for Pregabalin was 0.1 μ g/mL and the linear calibration curve was acquired with $r > 0.99$ between 0.1 and 20 μ g/ml. The intra-day and inter-day variation of the current assay was evaluated with the coefficient of variations within 10.73% at LLOQ and 9.8% for other quality control samples, whereas the mean accuracy ranged from 96.8% to 107.6%. The present method provides a robust, rapid and sensitive analytical tool for Pregabalin in human plasma and has been successfully applied to a clinical pharmacokinetic study where Pregabalin was orally administered in healthy subjects.

INTRODUCTION

Pregabalin (PRG) is an amino acid derivative of gamma-amino butyric acid (GABA analogue). PRG is the pharmacologically active S-enantiomer of 3-amino methyl-5-methyl-hexanoic acid (Frampton and Foster, 2005). It is an antiepileptic agent licensed for adjunctive therapy in the treatment of partial seizures and for peripheral neuropathic pain (Blommel and Blommel, 2007).

The exact mechanism of action of PRG is unclear, although it may reduce excitatory neurotransmitter release by binding to the alpha 2-delta protein subunit of voltage-gated calcium channel (Frampton and Foster, 2005). Compared to other GABA analogues only PRG has been approved by FDA for both the management of diabetic peripheral neuropathy and post herpetic neuralgia.

* Corresponding Author

Address: Panacea Biotec Ltd, 72/3 TTC Industrial Area, Mahape, Navi Mumbai, Maharashtra -400710, India.

Contact: +91-22-67333000; Fax: +91-22-6733 3111

Various methods have been published for the estimation of PRG in biological fluids. They include pre column derivatisation followed by HPLC-fluorescence detection (Vermeij and Edelbroek, 2004) protein precipitation followed by liquid chromatography mass spectrometry (LC-MS/MS) detection (Berry et al., 2007; Adler et al., 2007; Mandal et al., 2008; Vaidya et al., 2007) liquid-liquid extraction with LC-atmospheric pressure chemical ionization (APCI) MS/MS detection (Nirogi et al., 2009; Shah et al., 2010) or derivatising reaction followed by Gas chromatographic (GC) determination (Thejaswini et al., 2012).

A much commonly used and simple ionization technique for LC-MS/MS is ESI compared to APCI method (Nirogi et al., 2009) and also an advantage of voltage is obtained with ESI.

A sample preparation technique using solid phase extraction gives a cleaner sample with no significant matrix effect compared to protein precipitation and liquid-liquid extraction techniques reported earlier (Berry et al., 2007; Adler et al., 2007; Mandal et al., 2008; Vaidya et al., 2007; Nirogi et al., 2009; Shah et al., 2010).

The advanced development in solid phase extraction field which uses polymer cartridges gives consistent and reproducible results compared to the liquid-liquid extraction or protein precipitation methods reported earlier. Also MS/MS detection enables a more sensitive method (LLOQ 100ng/ml) compared to the GC detection with sensitivity of 2µg/ml as reported previously (Thejaswini *et al.*, 2012).

The aim of the present study was to develop and validate a sufficiently sensitive, precise, accurate and fast UPLC–MS/MS (MRM) method for the quantification of PRG in human plasma to be applied in a pharmacokinetic study to show the reliability of the assay in samples from humans, who were subjected to 300 mg PRG oral dose.

MATERIALS AND METHODS

Chemicals

PRG (Fig 1) was purchased from Symed Labs Ltd, India and PRG-d4 (Fig 1) from Varda Biotech (P) Ltd, India. Acetonitrile (Gradient grade for HPLC) was purchased from Sigma Aldrich, Ammonium acetate and formic acid (puriss grade) was purchased from Fluka, perchloric acid 70%, GR grade from Merck, Purified water obtained from Milli Q system was used throughout the experiment. All other reagents were of analytical reagent grade.

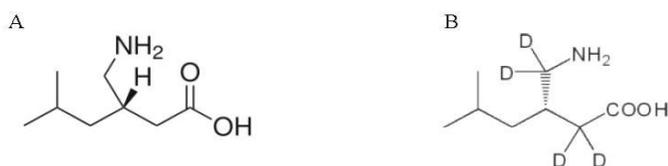


Fig. 1: Chemical structures of (A) Pregabalin and (B) Pregabalin-d4.

Instrument and Conditions

LC-MS/MS analysis was performed using Waters Acquity UPLC, coupled with Waters Quattro Premier XE tandem quadrupole mass spectrometer (Waters, USA). All data were acquired employing MassLynx quantitative analysis data processing software.

The separation was performed on Supelco, Ascentis Express RP amide fused core column (50 x 2.1mm, 2.7µm). The mobile phase was a mixture of Acetonitrile and 2mM ammonium acetate solution pH adjusted to 2.5 (80:20, v/v) pumped at a flow rate of 0.3 mL/min. the column temperature was maintained at 40°C. The detection was operated in electrospray positive ionization using multiple reaction monitoring mode and the transitions of m/z 159.77 → 54.84 for PRG and 163.86 → 145.95 for internal standard were monitored.

Standard Solutions

Stock Solution (1mg/mL) of PRG was made in methanol. Separate stock solutions were prepared for calibration standard and quality controls. Further intermediate solutions were prepared by serial dilutions of stock solution in diluent (50% methanol in

water). Working solutions for calibration and quality control samples were prepared by diluting these intermediate solutions with diluent.

These solutions were stored at 2-8°C, and found stable at least for 9 days under these conditions. The calibration standard and quality control plasma samples were prepared by addition of working solutions to drug-free plasma in volumes not exceeding 5% of the plasma volume. The solution of internal standard were obtained by dissolving PRG-d4 in methanol to a concentration of 1mg/mL and further diluting this solution to a concentration of 100 µg/mL with diluent.

Preparation of Samples

The plasma samples were stored at -80°C and thawed at room temperature before processing. 125µL of plasma sample were aliquoted to polypropylene tube, 5µL of internal standard solution was added and vortexed for 10secs. 125µL of 50% perchloric acid was added to each sample and vortexed at 70 rpm for 2 minutes. Added 250µL water to each sample and vortexed for 30 secs, centrifuged the sample and supernatant subjected to solid phase extraction (SPE) using Oasis HLB 30 mg/1ml SPE tubes. The sample supernatant was added to the pre-conditioned cartridge, further washed with water followed by 5% methanol and finally eluted with 90 % methanol.

The sample was evaporated to dryness under a gentle stream of nitrogen in a water bath at a temperature of 37 °C. The dried samples were then dissolved in 0.5 mL of mobile phase by vortexing and transferred to UPLC vials and a 1µL aliquot was injected into the chromatographic system.

Calibration Curve

The calibration curve was constructed in the range 0.1 to 20 µg/mL of PRG. The concentration of calibration samples for PRG are 0.1, 0.2, 1.4, 3.2, 10.0, 12.8, 15.2 and 20 µg/mL. The calibration curves were obtained by weighted linear regression (weighing factor 1/x): the peak area ratio (analyte/IS) was plotted vs the analyte concentrations. The suitability of calibration model was confirmed by back-calculating the concentration of the calibration standards.

Method Validation

The method validation was performed as per the guidelines for Bioanalytical Method Validation of Food and Drug Administration (CDER, May 2001). Full validation is performed in human plasma. Human plasma was spiked with standard solutions to obtain a calibration curve with eight different concentrations. The main QC concentrations quantified were 0.3 (low), 2.4 (medium-1), 8 (medium-2), 14 (high) µg/ml and 0.1 µg/ml as LLOQ (Limit of quantification).

Specificity of the method was assessed by comparing the chromatograms of six different lots of blank human plasma with those of corresponding spiked plasma sample at LLOQ level. Linearity was assessed by the correlation coefficient(r) and relative

error of calibration standard samples. Intra- and inter-day precision and accuracy were determined by analyzing six replicates of each QC level on 3 separate days, and on each day samples were analyzed together with an independently prepared calibration curve. The precision and accuracy were expressed as relative standard deviation (R.S.D.) and % assay respectively. The LLOQ was defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%.

The extraction recovery of PRG was determined at four QC levels ($n = 6$) by comparing the peak areas of PRG in the extracted QC samples with corresponding neat solutions of PRG. Matrix effect was evaluated by determining the precision of back calculated concentration of PRG at two concentration levels (low QC and high QC) in six different lots of matrix, against a single calibration curve.

Stability of PRG was evaluated in QC samples at room temperature after storing for 4 h, after three freeze–thaw cycles and after 60 days at -80°C . Stability of PRG in processed samples stored in auto sampler vials at room temperature for >24 h was also assessed.

Pharmacokinetic Study

The validated method described in this article was applied to estimate PRG in clinical study samples after oral administration of Pregabalin formulation. Thirty male healthy volunteers were used in the study.

All the volunteers were administered three doses of commercially available Pregabalin formulation 100mg at an interval of 8h. Blood samples were collected in EDTA vacutainer at pre dose and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 8, 10, 12, 16, 20, 24, 30, 36 and 48 h post dosing. The collected blood samples were centrifuged immediately at 3500 g for 10 minutes.

The supernatant was transferred to respectively labeled polypropylene tubes and stored at -80°C until analysis. This study protocol was approved by independent ethics committee and the study was conducted as per pertinent requirements of the current ICMR guidelines and USFDA adopted ICH guidelines for Good Clinical Practice and Declaration of Helsinki (Seoul, 2008) and informed consent from subjects were obtained prior to the commencement of study. The pharmacokinetic parameters were calculated using Winnonlin software (Ver. 5.3 Pharsight, USA).

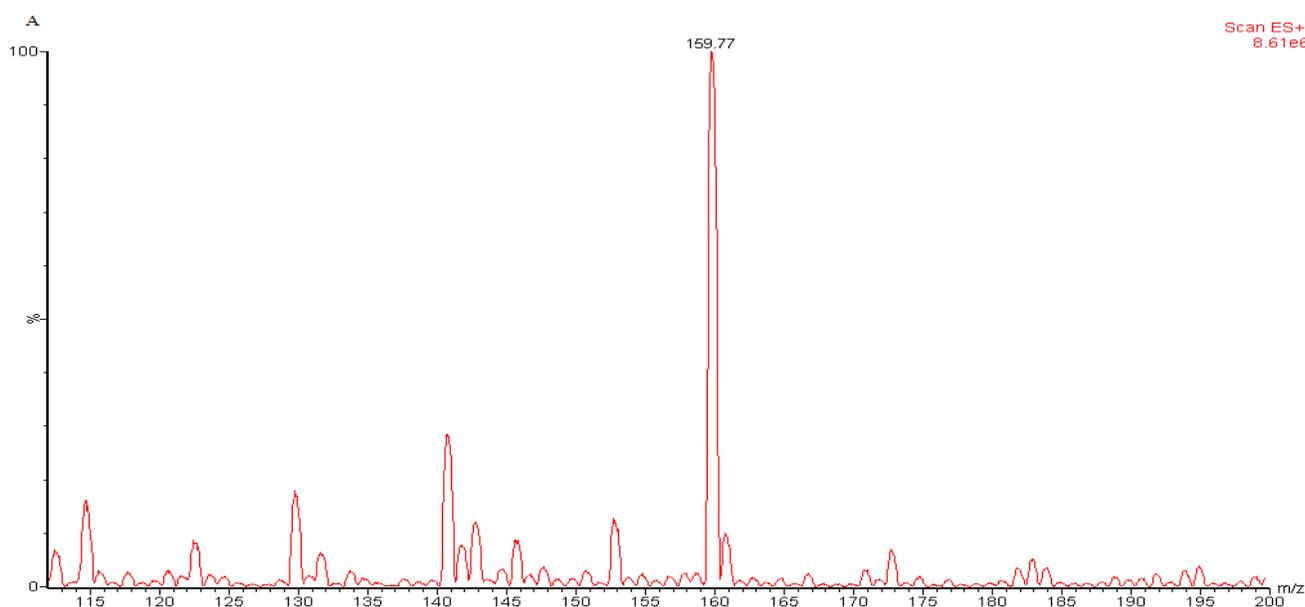
RESULTS AND DISCUSSION

Selection of Internal Standard

PRG-d4 was selected as internal standard for the assay as it is well separated from the analyte. It showed similar ionization efficiency and adequate, reproducible and similar extraction recovery from plasma employing the sample pre-treatment protocol described above. In addition, it is not an endogenously occurring substance.

Method development and optimization of MS/MS conditions

In order to develop the ESI MS/MS method for the determination of PRG, the full scan precursor and product ion spectra of the analyte (PRG) and the IS were acquired in positive ion mode by infusing each compound separately at a concentration of $0.1\ \mu\text{g}/\text{mL}$ in Methanol:water (50:50) at a flow rate of $5\ \mu\text{L}/\text{min}$. The scan gave rise to predominant $[\text{M}+\text{H}]^{+}$ ion peak at m/z 159.77 and 163.86 respectively for PRG and IS (Fig 2). Following detailed optimization procedure for fragmentation of the parent molecule, product ion spectra was obtained for PRG and IS (Fig. 2). The results showed that the most sensitive mass transition was m/z 159.77 \rightarrow 54.84 for PRG and m/z 163.86 \rightarrow 145.95 for the IS. These product ions were used for quantification.



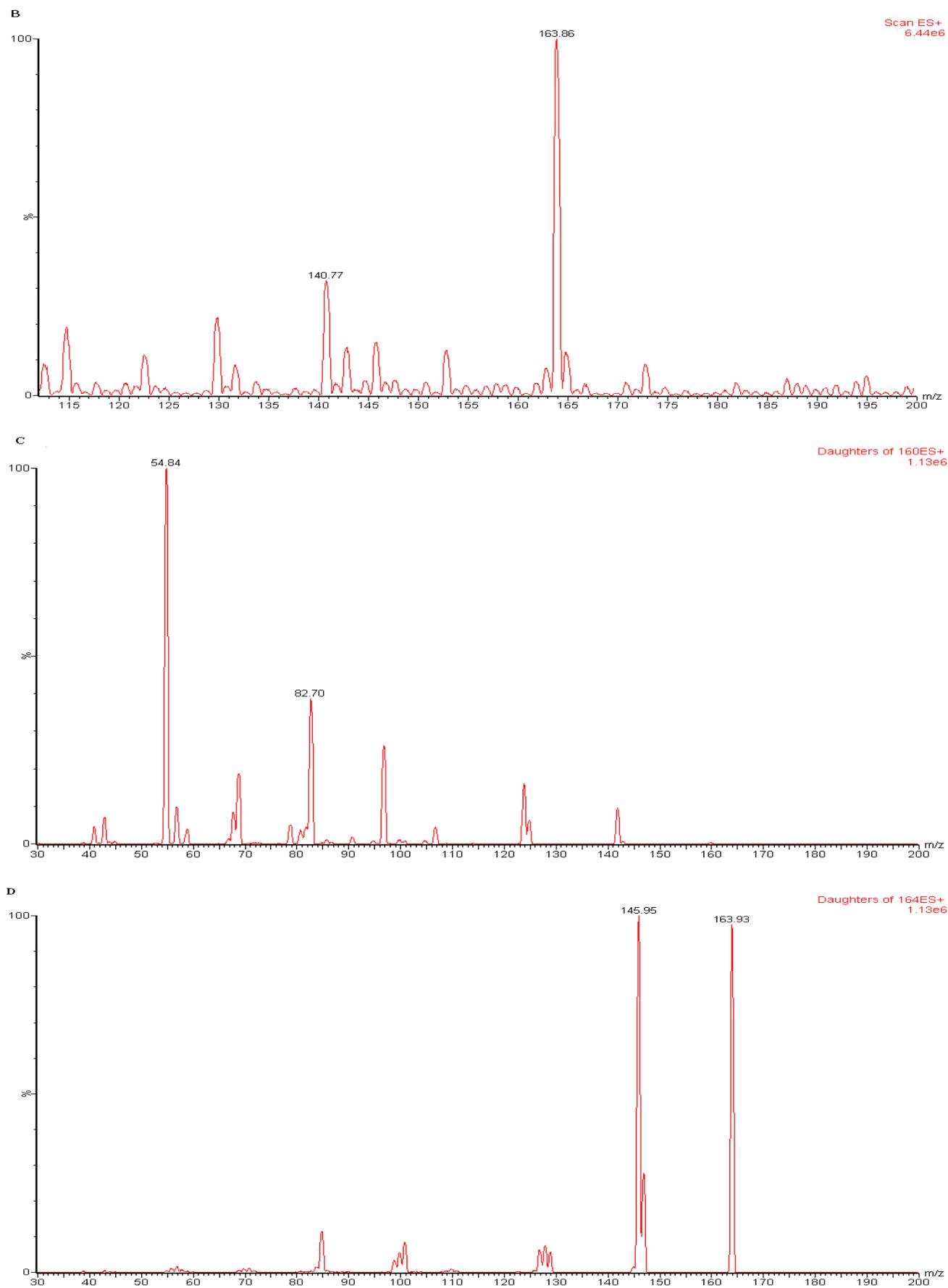


Fig. 2: Mass spectrum Pregabalin and IS: (A) full scan spectrum of Pregabalin; (B) full scan spectrum of IS (C) product ion spectrum of Pregabalin at m/z 159.8; (D) product ion spectrum of IS at m/z 163.9.

Liquid chromatographic conditions

The chromatographic conditions were optimized for the rapid and efficient separation of PRG and IS from plasma components. For this reason, a C18 stationary phase and Acetonitrile as the organic modifier of the mobile phase were chosen, over C8 and methanol respectively, as this combination gave the best results in terms of peak shape (width, symmetry, sharpness). Further optimization was performed by adding 0.1% FA in the mobile phase and increasing the column temperature, which improved even more the peak shape of the analyte, accelerated analysis time and enhanced ionization efficiency. Finally, under the optimized chromatographic conditions PRG eluted at 0.34 min and PRG-d4 (IS) at 0.34 min, respectively, as shown in Fig. 3.

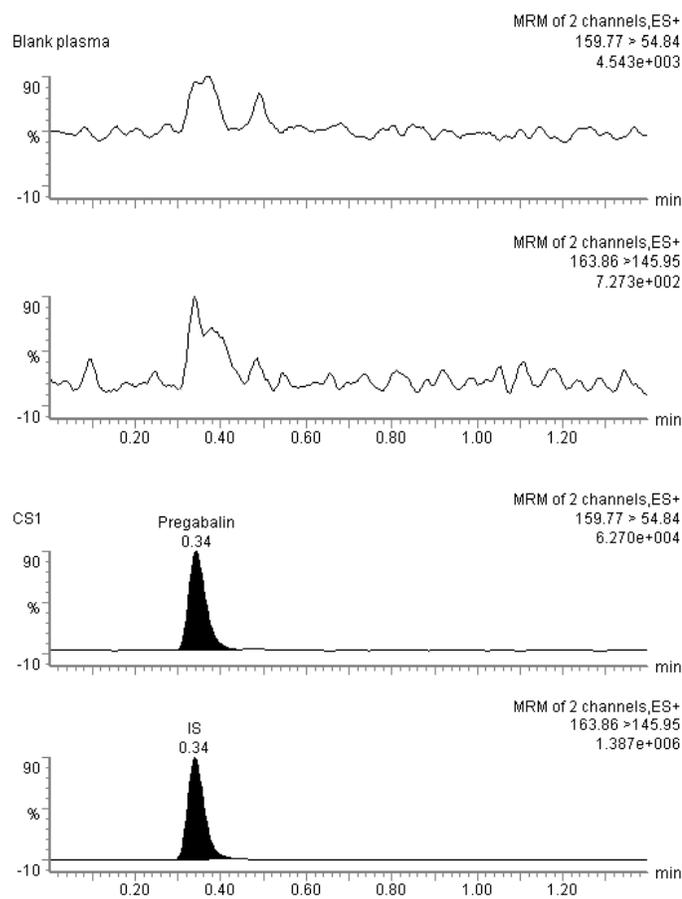


Fig. 3: Representation MRM chromatograms of Pregabalin and IS in human plasma: (A) blank plasma; (B) plasma sample spiked with Pregabalin (at LLOQ conc) and IS.

Sample Preparation

The solid phase extraction of plasma samples with Oasis HLB 30mg/1ml cartridge was selected over liquid-liquid and solid phase extraction for fast, simple and efficient sample clean-up. It was found that the protein precipitation by acid and further clean up by SPE provided almost quantitative recovery of PRG and no interferences from plasma matrix. Furthermore, due to its simplicity and speed, this sample pre-treatment protocol can be considered compatible with high throughput analysis.

Method Validation

Selectivity

The calibration standard plasma samples analyzed by the developed analytical methodology showed that the retention time (RT) of PRG and IS was 0.34 min and 0.34 min, respectively. All batches of blank drug-free human plasma analyzed under the same conditions were found to be free of interference peaks from endogenous plasma substances at the RT of the analyte and the IS, demonstrating the selectivity of the assay. Representative chromatograms of blank human plasma and spiked blank plasma with PRG at the LLOQ are shown in Fig. 3a and b, respectively.

Calibration Curve and LLOQ

Calibration curves were constructed by plotting the peak area ratios of PRG to IS of plasma calibration standards versus nominal concentrations of the analyte. The calibration model was selected based on the analysis of the data by linear and non-linear regression as well as with and without weighting. Calibration curves of five different lots of plasma calibration standards were constructed over the concentration range 0.1–20 µg/mL. The best linear fit and least square residuals for the calibration curves were achieved with 1/x weighting factor, giving a mean linear regression equation for calibration curve : $y = 0.138465 x + 0.004559$ where y is the peak ratio of the analyte to IS and x is the concentration of the analyte. The correlation coefficient (r) of the calibration curve was >0.999, indicating good correlation (Fig.4). The lower limit of quantification with the proposed method was 0.1 µg/mL (Table 1) with signal to noise ratio (S/N) > 5 (CDER, May 2001). This limit was sufficient for clinical drug monitoring after oral administration of 300 mg dose of Pregabalin. The mean accuracy and %CV (precision) for LLOQ were 106.3% and 10.73% respectively.

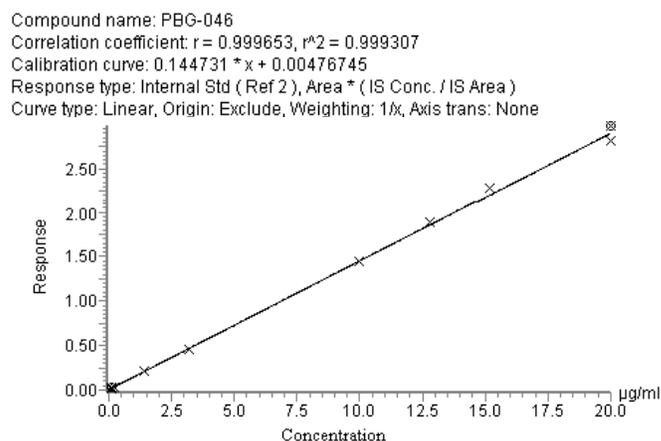


Fig. 4: Representative Calibration curve for validated range 0.1-20µg/ml.

Intra and Inter-day Precision and Accuracy

The accuracy and precision data for intra- and inter-day plasma samples are presented in Table 1. The assay values for both occasions (intra- and inter-day) were found to be within the accepted variable limits. The data indicated that the present method has a satisfactory accuracy, precision and reproducibility.

Recovery

The extraction recovery data (%CV) of Pregabalin from human plasma was calculated by analyzing six replicates at 0.3, 2.4, 8.0 and 14.0 ng/mL together with the recovery of the IS at the concentration of 100 µg/mL. The extraction recoveries of assay were 63.89, 63.44, 60.18, 54.78% respectively and 56.3% for IS. The global recovery found to be 60.6% with a precision of 6.9% (%CV). The data showed that the recovery is almost quantitative and consistent for both Pregabalin and IS.

Matrix Effect

In this study, the variability in matrix effect was evaluated by analysing the low (0.3µg/mL), and high (14.0µg/mL) QC samples. The % CV values are <15% for PRG at low and high QC levels in all the six matrix lots. The matrix effect on the ionisation of the analyte and IS was not obvious under these conditions.

Stability

Samples at low and high QC concentrations were analysed in six replicates for studying the possible conditions to which the samples might be exposed during storage and handling. The results of the investigated stability parameters (Table 2)

showed that stock solutions in methanol were stable for 9 days at -20°C. Plasma samples were stable for 4 h at room temperature and for 2 months at -80°C.

However, study samples may require re-analysis, which results in extra freeze/thaw cycles. Stability of PRG in plasma is adequate during three freeze (-80°C)/thaw cycles. The treated plasma samples were stable for 45h at auto sampler temperature (5°C). All the stability experiment results were found to be within the assay variability limits.

Application to the analysis of clinical study samples

The proposed analytical methodology was applied to the analysis of plasma samples obtained from 30 healthy human volunteers following oral administration of 3 x 100mg of Pregabalin marketed formulation as a part of pharmacokinetic study. The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of PRG in humans. Fig 5 depicts the mean plasma concentration vs time profile of PRG in these volunteers under fasting conditions. Following the oral administration of 300 mg of PRG formulation to the volunteers under fasting conditions, the mean plasma concentration (C_{max}) 3.14µg/mL, were attained at ~10h (T_{max}), while the $AUC_{(0-t)}$ was found to be 57.15µg.h/mL.

Table 1: Intra-day and Inter-day mean, standard deviation, accuracy and precision for Pregabalin.

Nominal Conc	Intra-day (n=6)				Inter-day (n=18)			
	Mean µg/mL	SD ^a µg/mL	% Accuracy	Precision (%CV)	Mean µg/mL	SD ^a µg/mL	% Accuracy	Precision (%CV)
0.3	0.309	0.007	102.5	2.28	0.308	0.023	102.4	7.47
2.4	2.456	0.098	102.2	4.00	2.440	0.145	101.5	5.95
8	8.542	0.284	106.6	3.33	8.621	0.546	107.6	6.33
14	13.579	0.401	96.8	2.96	14.434	1.413	102.9	9.79

^aSD – standard deviation

Table 2: Stability of Pregabalin under different conditions (n=6)

Concentration	Room Temp 4hr	Freeze thaw 3 cycles (-80°C)	Auto-sampler (5°C) 45hr	Long term 60days (-80°C)
0.3	94.2%	91.0%	103.6%	87.4%
14.0	102.8%	92.7%	100.6%	105.9%

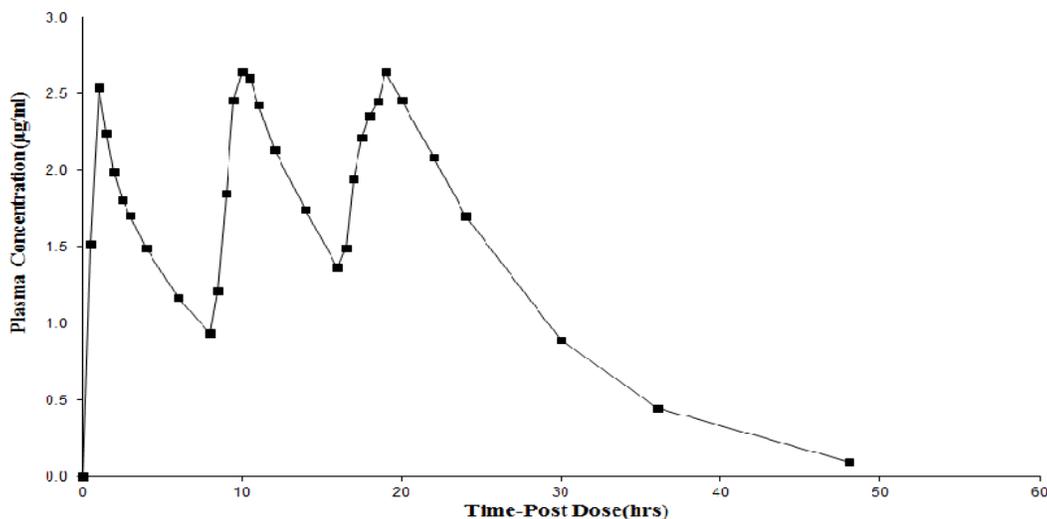


Fig. 5: Mean plasma concentration–time curves of Pregabalin after administration of Pregabalin oral formulation 3x 100 mg (tid).

CONCLUSION

The described UPLC–ESI MS/MS validated analytical methodology enables the rapid and selective assay of Pregabalin in human plasma. The proposed method presents high sensitivity, accuracy, precision, recovery and stability combined with high accuracy mass measurement, thus being suitable for monitoring of pharmacokinetic, bioavailability and bioequivalence studies. The simplicity of the sample pre-treatment and the rapid analysis time could render this method as an ideal high-throughput method for the efficient analysis of a large number of clinical samples.

ACKNOWLEDGEMENTS

The authors wish to thank the management of Panacea Biotech Ltd, India for permitting this work to be published.

REFERENCES

- Adler RS, Carter SJ, Voelker T. Overcoming challenges in development of LC/MS/MS methods for highly polar, low molecular weight, zwitterionic compounds: a quantitative assay for Pregabalin. *Tandem Labs* (2007); [ONLINE] Available at: www.tandemlabs.com/capabilities_publications. [Accessed 14 March 2012].
- Blommel ML, Blommel AL. Pregabalin: An Antiepileptic Agent Useful for Neuropathic Pain. *American Journal of Health-System Pharmacy*, 2007; 64(14):1475-1482.
- David Berry, Gar eth WH, Lisa JC, Donald PC. Therapeutic Drug Management of Antiepileptic Drugs- Multidrug Analysis using UPLC-MS/MS. Waters corporation, ArticleNo:720002302en.(2007); [ONLINE] Available at: <http://www.waters.com/webassets/cms/library/docs/720002302en.pdf>. [Accessed 10 April 2012].
- Frampton JE, Foster RH. Pregabalin: in the treatment of postherpetic neuralgia. *Drugs*, 2005;65(1):111-120.
- Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), May 2001. Available at: <http://www.fda.gov/cder/guidance/index.htm>. [Accessed 14 March 2012].
- Mandal U, Sarkar AK, Gowda KV, Agarwal S, Bose A, Bhaumik U, Ghosh D, Pal TK. Determination of Pregabalin in Human Plasma Using LC-MS-MS. *Chromatographia*, 2008; 67(3-4): 237-243.
- Nirogi R, Kandikere V, Mudigonda K, Komarneni P, Aleti R. Liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry method for the quantification of pregabalin in human plasma. *J Chromatogr B*, 2009; 877: 3899 – 3906.
- Shah GR, Ghosh C, Thaker BT. Determination of pregabalin in human plasma by electrospray ionisation tandem mass spectrometry. *J Adv Pharm Tech Res*, 2010; 1(3): 354–357.
- Thejaswini JC, Gurupadayya BM, Raja P. Gas Chromatographic Determination of Pregabalin in Human Plasma using Ethyl Chloroformate Derivatizing Reagent. *Journal of Pharmacy Research*, 2012; 5(6): 3112.
- Vaidya VV, Yetal SM, Roy SMN, Gomes NA, Joshi SS. LC-MS-MS Determination of Pregabalin in Human Plasma. *Chromatographia*, Dec 2007; 66(11-12):925-928.
- Vermeij TAC, Edelbroek PM. Simultaneous high-performance liquid chromatographic analysis of pregabalin, gabapentin and vigabatrin in human serum by pre-column derivatization with o-phthalaldehyde and fluorescence detection. *J Chromatogr B*. 2004;810: 297-303.

How to cite this article:

Anju Aji, Sarita Karthikeyan, Sarabjit Singh, Shivanand P. Puthli. A Novel UPLC–MS/MS Method for Determination of γ -Amino Butyric acid Analogue in Human Plasma: Application to Pharmacokinetic Study. *J App Pharm Sci*. 2013; 3 (06): 172-178.