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RESEARCH ARTICLE

LC-MS-MS METHOD VALIDATION OF PREGABALIN IN HUMAN PLASMA

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A rapid, sensitive and specific method for quantification of pregabalin in human plasma using imipramine as internal standard was validated. Sample preparation involved a solid phase extraction procedure. The extract was analyzed by high performance liquid chromatography coupled to electrospray tandem mass spectrometry (LC-MS-MS). Chromatography was performed isocratically on a Hypurity, 5 μm C-18 (50 × 4.6 mm *i.d.*), analytical column with buffer-methanol 20:80 (*v/v*) as mobile phase. The response to pregabalin was a linear function of concentration over the range 250.00 to 20000.00 ng/ml. The lower limit of quantification in plasma was 250.00 ng/ml. The method was successfully applied in a bioequivalence study of a pregabalin formulation after administration single oral dose.

Key words: Pregabaline, Method validation, LC-MS-MS, Human plasma.

INTRODUCTION

Pregabalin has the molecular formula $C_8H_{17}NO_2$ and its chemical name is (3*S*)-3-(aminomethyl)-5-methylhexanoic acid (**Figure 1**) with molecular weight 159.23 (Baber, 1994; US FDA, 2000).

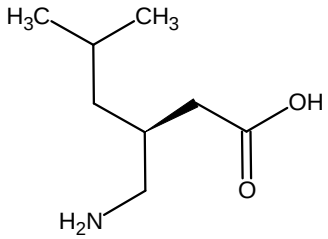

Fig. 1. Structure of Pregabalin

Pregabalin is a white to off-white, crystalline solid with a pK_{a1} of 4.2 and a pK_{a2} of 10.6. It is freely soluble in water and both basic and acidic aqueous solutions. Pregabalin binds with high affinity to the alpha$_2$-delta site (an auxiliary subunit of voltage-gated calcium channels) in central nervous system tissues. *In vitro*, pregabalin reduces the calcium-dependent release of several neurotransmitters, possibly by modulation of calcium channel function. Pregabalin is a structural derivative of the inhibitory neurotransmitter gamma-amino butyric acid (GABA). Pregabalin increases the density of GABA transporter protein and increases the rate of functional GABA transport (Crofford *et al* 2005). The literature is enriched with several findings on analytical method validation studies of drugs in human plasma (Zhao *et al* 2009; Ptacek *et al* 2012; Basaveswara Rao *et al* 2012). In present investigation, the objective is to validate bioanalytical parameters, system suitability, linearity curve, percent recovery, inter-day and intra-day precision and accuracy, stability, dilution integrity, partial volume, matrix effect, reinjection reproducibility for pregabalin in human plasma.

EXPERIMENTAL

Chemicals and reagents

Working standard of pregabalin was obtained from Cadila Health Care Ltd., Ahmedabad.

Working standard of imipramine was obtained from Sigma-Aldrich Pvt. Ltd., Bangalore. Methanol HPLC grade was from J.T. Baker. Hexane and water (HPLC grade), ortho-phosphoric acid, glacial acetic acid were from Merck India Pvt. Ltd., Bangalore. Ammonium acetate ACS grade was from Sigma-Aldrich Corporation, Bangalore.

Instrumentation and chromatographic conditions

The HPLC system (Shimadzu) consisted of a binary pump and an auto sampler. Detection was performed with an Applied Biosystems MDS Sciex (API 2000) mass spectrometer with atmospheric ion spray for ion production, which was controlled by Analyst 1.4.2 software. Chromatography was performed isocratically on a 50 mm × 4.6 mm *i.d*., 5 μm particle, hypurity C18 analytical column.

The mobile phase was buffer-methanol 20:80 (*v/v*) at a flow rate of 0.9 ml/min with splitter. Buffer was 5 mM ammonium acetate, pH adjusted to 3.5 with glacial acetic acid. Chromatography was performed at ambient temperature. The instrument was set up in the multiple reactions monitoring (MRM) positive mode.

Preparation of stock solution, calibration and quality control samples

A stock solution of pregabalin (1000.00 μg/ml) was prepared in methanol. A stock solution of imipramine (1000.00 g/ml) was prepared in mobile phase. A series of working standard of pregabalin containing 50, 500 and 1000 μg/ml was prepared by diluting the stock solution with diluent methanol:water (60:40) *v/v*. Working internal standard solution containing 7, 100 and 1000 μg/ml was prepared in methanol: water (60:40) *v/v*.

Low, medium, and high concentration quality control solutions (750.00, 7000.00, 10000.00 and 17250.00 ng/ml, respectively) were prepared in diluents methanol:water (60:40) *v/v*. Calibration plot standards were prepared by spiking blank plasma with pregabalin at concentration of 250, 500, 1000, 2000, 4000, 8000, 12000, 16000 and 20000 ng/ml.

Quality control samples were prepared by spiking blank plasma with 250.00, 750.00, 7000.00, 10000.00, 17250.00 and 20000.00 ng/ml pregabalin. The stock solutions were stored at 2-8 °C and used within 33 days of preparation.

Validation procedures

The method was validated in accordance with current acceptance criteria. The system suitability was done by injecting six replicates of M1 quality control samples in LC-MS-MS. The acceptance criterion for system suitability was that the mean %RSD of pregabalin/imipramine area ratio was NMT 5%. The specificity of the analytical method was investigated by extraction and analysis of blank plasma samples from six different sources to assess potential interference from endogenous substances. The apparent response at the retention times of pregabalin and imipramine was compared with that at the lower limit of quantification (250.00 ng/ml). The acceptance criterion for pregabalin was that the mean interference from the six individual sources should be $< 20\%$ of the signal at the LLOQ and that the mean interference for imipramine (internal standard) from the six individual sources should be $< 5\%$ of the signal at working concentration.

The calibration equation was determined by least-squares linear regression over the range 250.00 to 20000.00 ng/ml in plasma (Adler *et al* 2007). The precision and accuracy of the methods were determined at the six QC sample levels for six replicates together with calibration samples from two sets (inter day and intraday) validation batches. The basic fundamental properties studied during method validation were the stability of stock solution stored at 2-8 °C for one month, freeze thaw stability through three cycles, short term stability, autosampler stability and long term stability. For freeze thaw stability, QC plasma samples were subjected to three cycles from -20°C to room temperature. Short term bench top stability was determined by placing samples on the bench top at ambient temperature for 24 h. An autosampler stability was assessed by placing processed QC samples in an auto sampler at 10°C for 24 h and long term stability was evaluated by freezing QC samples at -20°C for a month. Recovery was assessed by comparing the peak areas of neat analyte standards with those for spiked standards at three concentrations before and after extraction.

Dilution integrity was performed to handle out of expectation values lying outside calibrated concentration range. 0.250 ml of pooled blank plasma was added to 0.250 ml of above spiked sample to prepare 1/2 dilution samples. 0.375 ml of pooled blank plasma was added to 0.125 ml of above spiked sample to prepare 1/4

dilution samples. Matrix effect was checked by processing six lots of plasma samples in duplicate. Low and high quality control stock solutions were spiked post extraction in singlet (100% recovery). Similarly, internal standard was also spiked post extraction. These quality control samples were injected along with calibration curve standards. After completion of run, the system was put in standby mode for at least 30 minutes and the instrument was re-equilibrated. These quality control samples were injected along with calibration curve standards processed using full volume and the concentration was calculated using dilution factor as 2 for 50% processing volume and 4 for 25% processing volume. Carry over effect was carried out by injecting samples in the sequence of extracted blank matrix sample with lower and upper limit of quantification samples in the duplicate (US EPA, 1995; Green, 1996; Vessman, 1996; Seno *et al* 1997; Winslow and Meyer, 1997; Krause, 2006).

RESULTS AND DISCUSSION

The assay was found to be linear for pregabalin concentrations in the range 250.00 to 20000.00 ng/ml. The values of correlation coefficients, slopes and intercepts are summarized in **Table 1** with an average value of r=0.9986.

Table 1. Parameters of linearity studies

	Correlation coefficient	Slope (m)	y–Intercept (c)
1	0.9997	0.0000477	0.0002430
2	0.9976	0.0000480	0.000246
3	0.9987	0.0000527	-0.0004920
4	0.9989	0.0000377	-0.0021100
5	0.9982	0.0000433	0.0004600
Mean	0.9986	0.0000459	-0.0003

Precision and accuracy were found to be satisfactory at six QC concentrations. The inter day precision and accuracy of the method at QC levels (250.00, 750.00, 7000.00, 10000.00 17250.00 and 20000.00 ng/ml) were 8.43, 6.69, 8.50, 9.14, 11.07 and 8.98% respectively. The intra day precision and accuracy of the method at QC levels (*n*=6) were 9.88, 5.45, 5.07, 4.73, 4.80 and 4.21% respectively. Results are summarized in **Table 2** and **Table 3**.

Table 2. Calibration standard in human plasma

	Mean*	%CV	Nominal*
Level 1	249.675	2.676	100.057
Level 2	501.938	6.452	100.576
Level 3	983.024	3.199	98.487
Level 4	1980.108	6.980	99.191
Level 5	4076.194	3.859	102.096
Level 6	8061.124	3.403	100.953
Level 7	12109.337	1.999	101.100
Level 8	16097.162	4.158	100.796
Level 9	19396.568	4.956	97.164

*ng/ml

The extraction recovery of the method at QC levels (*n*=6) was 60.81, 53.23, 56.90 and 53.27% respectively. Absolute mean recovery of pregabalin and internal standard (imipramine) were 56.05 and 49.77% respectively. Stock solution stored at 2–8°C was found to be stable for 33 days. When drug stability at the LQC and HQC concentrations was measured after three freeze thaw cycles, the differences from freshly prepared samples LC-MS-MS method for determination of pregabalin in human plasma was low with values 7.85 and -1.85% respectively. When bench top stability at the LQC and HQC concentrations for 24 h was measured the differences from freshly prepared QC samples were 4.43 and -0.94% respectively.

Table 3. Data for inter day and intra day precision and accuracy

	Inter day			Intra day		
	Mean*	%CV	% Nominal*	Mean*	%CV	% Nominal*
LLOQ	259.385	8.43	103.89	237.056	9.88	94.95
LQC	729.526	6.69	97.40	726.144	5.45	96.95
MQC	7010.709	8.50	100.28	6784.305	5.07	97.05
M1QC	9916.235	9.14	99.29	9581.753	4.73	95.94
HQC	17382.117	11.07	100.90	15871.313	4.80	92.13
ULOQ	20308.206	8.98	101.93	19019.226	4.21	95.46

*ng/ml

When auto sampler stability at the LQC and HQC concentrations for 24 h was measured, the differences from freshly prepared samples were approximately 4.71 and 11.39% respectively. When drug stability in the matrix at -20°C for 31 days was measured at the LQC and HQC concentrations difference from freshly prepared samples were 6.08 and 5.34% respectively. Batch precision of quality control having same dilution factors (50% dilution) and (25% dilution) were 5.35 and 3.52%. The low and high quality control sample for reinjection reproducibility were 6.03 and -2.0%. %CV for partial volume was 5.35 and 3.52%.

CONCLUSION

The method for estimation of pregabalin from human plasma by LC-MS/MS was validated for all parameters like system suitability, selectivity and sensitivity, linearity curve, percent recovery, precision and accuracy, bench top stability, freeze thaw stability, auto sampler stability, stock solution stability, dilution integrity, carryover, partial volume, matrix effect, reinjection reproducibility as per standard operating procedure. The results obtained during the validation of the method fulfilled all requirements and recommendations, generally accepted for bioanalytical studies.

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