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Research Article

LC-MS-MS METHOD FOR THE DETERMINATION OF PREGABALIN IN HUMAN PLASMA.

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ABSTRACT

A bioanalytical method has been developed and validated for determination of Pregabalin in human plasma. The analytical method consists in the precipitation of plasma sample with trichloro acetic acid (20% v/v solution in water), followed by the determination of Pregabalin by an LC-MS-MS method using Tramadol as internal standard. Solid phase extraction was carried out using Strata-X 33 µm cartridges. Separation was achieved on a Kromasil 100 C_{18} (3.5 µm, 3, 30 mM) column with a mobile phase consisting of Acetonitrile-0.5% formic acid (80:20). Protonated ions formed by a turbo ionspray in positive mode were used to detect analyte and internal standard. The MS-MS detection was by monitoring the fragmentation of 160.2 (parent) 55.1 (product) (m/z) for pregabalin and 264.2 (parent) 58.1 (product) (m/z) for tramadol on a triple quadrupole mass spectrometer. The assay was calibrated over the range 50.00 to 8003.55 ng/ml with correlation coefficient of 0.9949. Validation data showed that within-run and between-run accuracy and precision, to be within the limits. No matrix effect was found in different sources of human plasma tested. Mean extraction recovery of pregabalin was 92.11% and 98.43% for IS. Plasma samples were stable for three freeze–thaw cycle at -70 °C.

Keywords: Pregabalin, Human plasma, LC-MS-MS.

INTRODUCTION

The reported methods for the estimation of drugs in plasma includes RP-HPLC and LCMS.[1, 2] Pregabalin (PGB) chemical name is (S)-3-aminomethyl-5- methyl hexanoic acid. It is a structural analogues of gamma aminobutyric acid (GABA). 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. The log of the partition coefficient (n-octanol/0.05M phosphate buffer) at pH 7.4 is - 1.35. Like gabapentin, pregabalin binds to the α2δ (alpha2delta) subunit of the voltage-dependent calcium channel in the central nervous system. This reduces calcium influx into the nerve terminals. Pregabalin also decreases the release of neurotransmitters such as glutamate, noradrenaline, and substance P (Australian Medicines Handbook). Pregabalin increases neuronal GABA levels by producing a dose-dependent increase in glutamic acid decarboxylase activity. Glutamic acid decarboxylase (GAD) is the enzyme that converts the excitatory neurotransmitter glutamate into the inhibitory GABA in a single step. For this reason, pregabalin greatly potentiates benzodiazepines, barbiturates & other depressants. For the determination of Pregabalin HPLC- UV, LC-MS / MS, and GC-MS [3-6] methods have been reported. Some of these methods use complicated extraction instruments, long and tedious extraction procedures, and large amounts of solvents or biological fluids for extraction. The main objective of this work is to develop rapid, selective and sensitive LC-MS / MS methods that have short and simple extraction procedures, consume small amounts of solvent and biological fluid for extraction and a short turn-around time.

MATERIALS AND METHODS

Chemicals and reagents

Methanol and Acetonitrile of HPLC grade from Merck, Water (HPLC Grade), Ammonium Formate, Trichloro acetic acid, Dichloromethane, n-Hexane, Diethylether and Formic Acid were AR Grade, Pregabalin and Tramadol from Aarti Drugs limited.

Instrumentation and chromatographic conditions

The mass spectroscopic detection was performed on Triple Quad 6460 (Agilent technologies) equipped using electrospray positive ionization. The chromatography was performed on a Kromasil 100 C_{18} (3.5 µm, 3, 30 mM). The mobile phase composition was a mixture of Acetonitrile and 0.5% formic acid (80:20) solution which was pumped at a flow rate of 1 mL / min with injection volume of 2 µL. Chromatograms were acquired using the computer based Analyst Software version 1.4.2.supplied by applied bioscience and the data were processed by peak area ratio. The ion spray potential was set at 3500 kV and the source of temperature was 300°C. The collision activation dissociation (CAD) gas setting at 10; Nitrogen was used as collision gas. The mass spectrometer was used in positive ion mode and multiple reaction monitoring (MRM) using turbo ion spray ionization mode as an interface. The transition m/z 160→255.1 was monitored for pregabalin and the transition m/z 264.2→ 58.1 for tramadol on a triple quadrupole mass spectrometer. Figure 1 and Figure 2 shows MS-MS scan for pregabalin and tramadol.

Preparation of calibration curve (CC) standards and quality control (QC) samples

A stock solution of pregabalin (1016.076ng/ml)and tramadol (505.14ng/ml) were prepared in methanol- water 80:20 (v/v). Calibration standards were prepared by spiking blank plasma with pregabalin to get the concentration of 50, 100, 375, 750 ,2064, 4128, 6070, 8550, 10000 ng/ml.Quality control samples were prepared by spiking blank plasma with 50, 128, 3200, 8000 ng/ml of pregabalin. The stock solutions were stored at 4-8° C.

Sample preparation

The cartridges were conditioned using 1ml of 100% methanol. It was acidified with 1 ml of 1% formic acid. Then 550µl sample (Analyte + IS) was added to it. It was washed twice with 1 ml of 1% formic acid. Finally it was eluted with 500µl of mobile phase (Acetonitrile: 0.5% Formic Acid) vortexes for 1 minute and 2 µL of the sample was injected.

Validation procedures

Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use[7-8]. The present method was validated in accordance with current acceptance criteria. Selectivity was assessed by analyzing blank plasma samples obtained from six different sources with six samples at LLOQ concentrations spiked using the biological matrix of any one source. Randomly selected blank human plasma sources were taken to determine the extent to which endogenous human plasma interfere with the analyte or the internal standard. No significant interference was observed in six different sources of human plasma samples. Representative chromatogram illustrating the specificity of the method are shown in Figure 3 and Figure 4**.** The calibration equation was determined by least squares linear regression (weighting $1/x^2$) over the range 50 to 10000 ng/ml in plasma**.** Assay precision and accuracy (inter-day and intra-day) values were determined across three precision and accuracy batches by analyzing six replicates each of LOQQC, LQC, MQC, and HQC samples in each batch. One of

the precision and accuracy batch was performed by different analyst to ensure ruggedness of the method. The stabilities were assessed under varying storage and handling conditions and determined by calculating the percentage nominal of LQC and HQC samples against freshly prepared calibration curve standards and compared with bulk spiked comparison samples (CS). As a part of method validation freeze thaw, bench-top, short term stability, long term stability was also evaluated. Recovery of the developed method can be evaluated by analyzing six replicates of analyte along with internal standard by comparing the analytical results for extracted samples at three concentrations (equivalent to LQC, MQC and HQC) with unextracted samples that represent 100% recovery. The percentage recovery of analyte and internal standard (IS) were calculated using appropriate chromatographic conditions.

RESULTS AND DISCUSSION

The assay was found to be linear for pregabalin concentrations in the range 50 to 10000 ng/mL . The results are presented in Table 1. The precision and accuracy were studied satisfactory at four QC concentrations for pregabalin. The intraday precision and accuracy of the method at QC levels (50.00 ng/ml, 128.057 ng/ml, 3201.419 ng/ml and 8003.548 ng/ml, n = 6) were 6.766, 1.255, 1.944 and 5.555% and 109.06, 98.97, 108.20 and 101.55 respectively. The interday precision and accuracy of the method at QC levels (n=6) were 2.307, 0.141, 0.985 and 1.714 % and 111.42, 90.62, 108.12, and 107.207 respectively. The results obtained from measurement of linearity, precision and accuracy are listed in Table 2 and 3. The results of stability studies of Pregabalin were presented in Table 4 showed that no significant degradation was observed under the test conditions which indicates that compounds are highly stable in plasma. The values obtained for the stability studies are within the acceptance criteria. Recovery of Pregabalin was evaluated by comparing mean analyte responses of six processed samples of low (LQC), medium (MQC) and high (HQC) quality control samples to mean analyte responses of six appropriately diluted pure diluted solutions. Mean recovery values are 88.92, 88.25 and 84.81 % at low, medium and high quality control levels respectively. Global mean recovery of Pregabalin was 92.11%. Mean recovery value for the internal standard was 89.43% and it is within the limit.

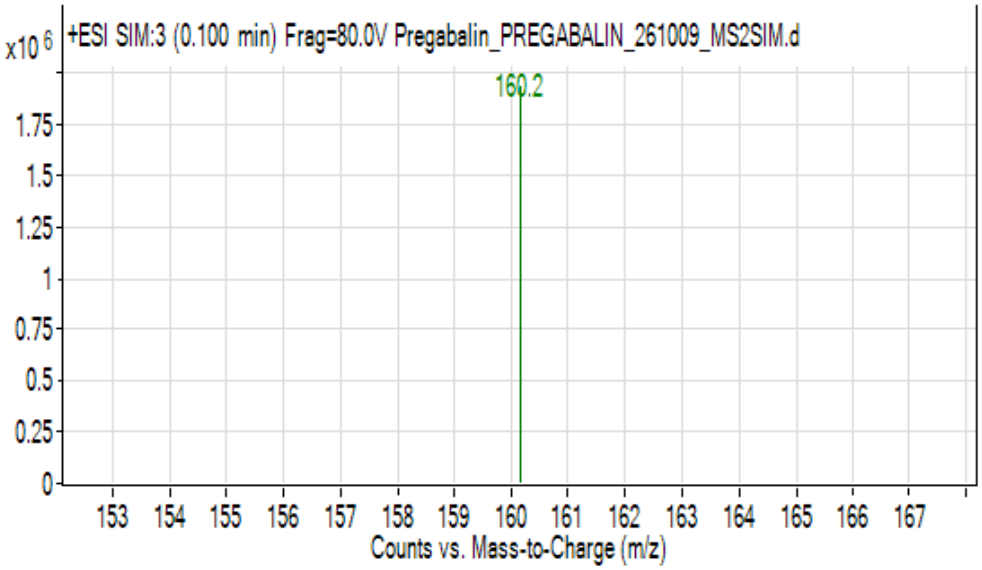

Fig. 1: Pregabalin Q1 MS-MS scan (160.20)

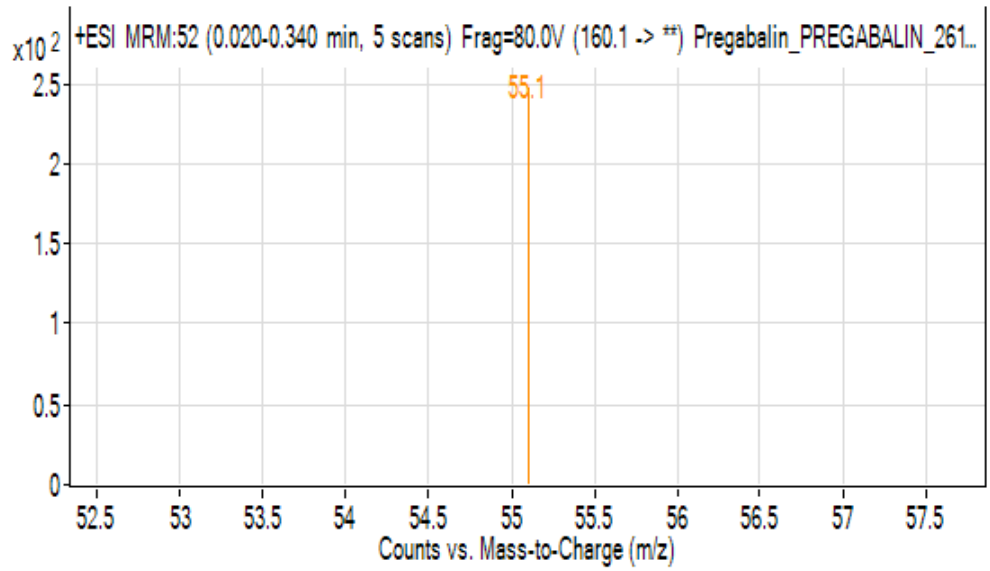

Fig. 1a: Pregabalin Q3 MS-MS scan (55.10)

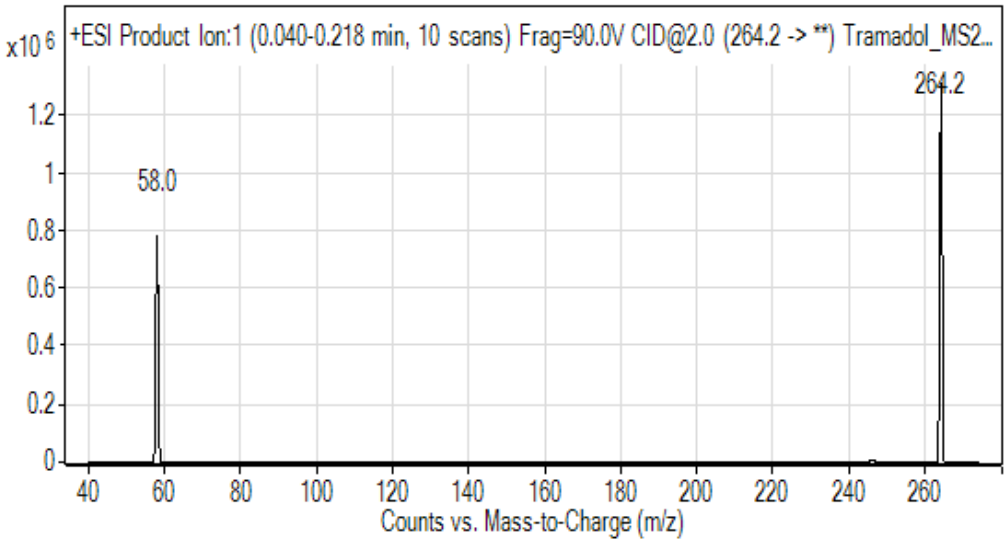

Fig. 2: Tramadol Q1 MS-MS scan (264.20)

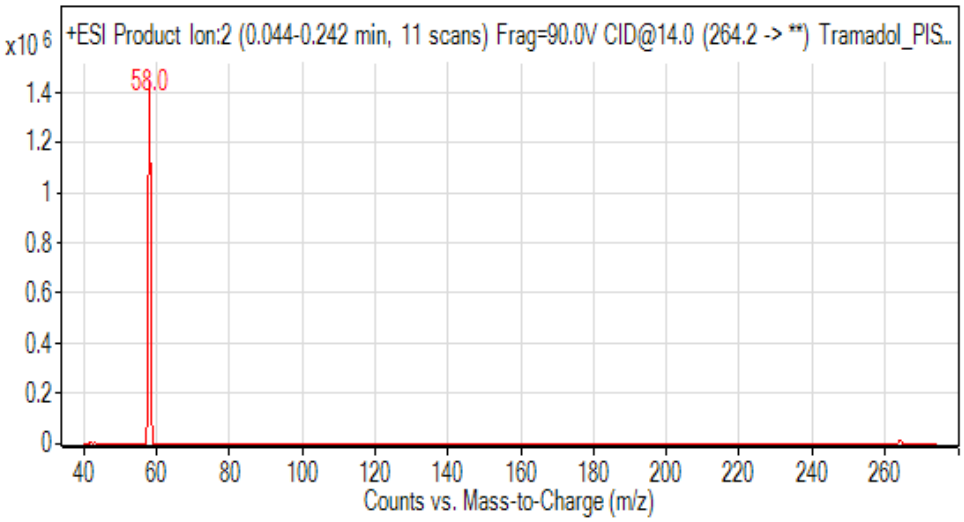

Fig. 2a: Tramadol Q3 MS-MS scan (58.0)

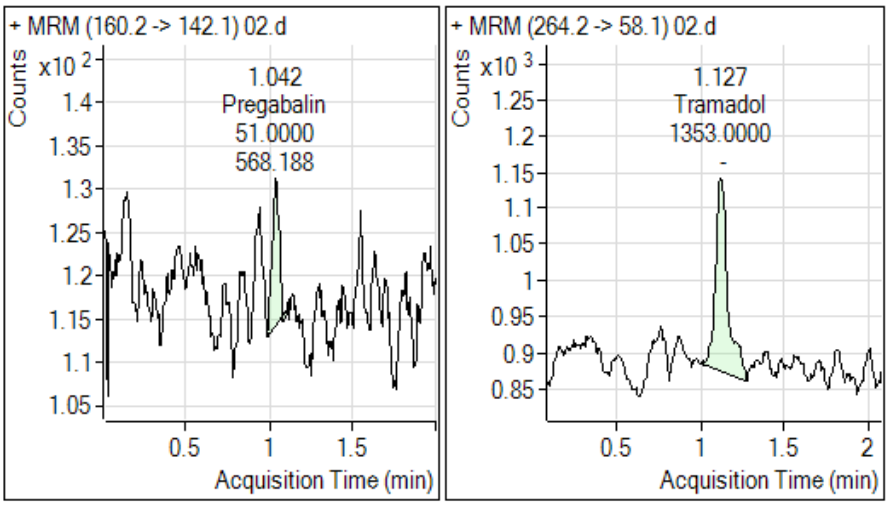

Fig. 3: Representative Chromatogram plasma blank for pregabalin and Tramadol

Table 1: Correlation coefficient (r)

Curve No.	Intercept	Slope	Correlation coefficient(r^2)
1	0.275	0.0007	0.9974
2	0.981	0.0006	0.9958
3	1.024	0.0001	0.9941
4	0.289	0.0005	0.9953
5	0.265	0.0004	0.9962

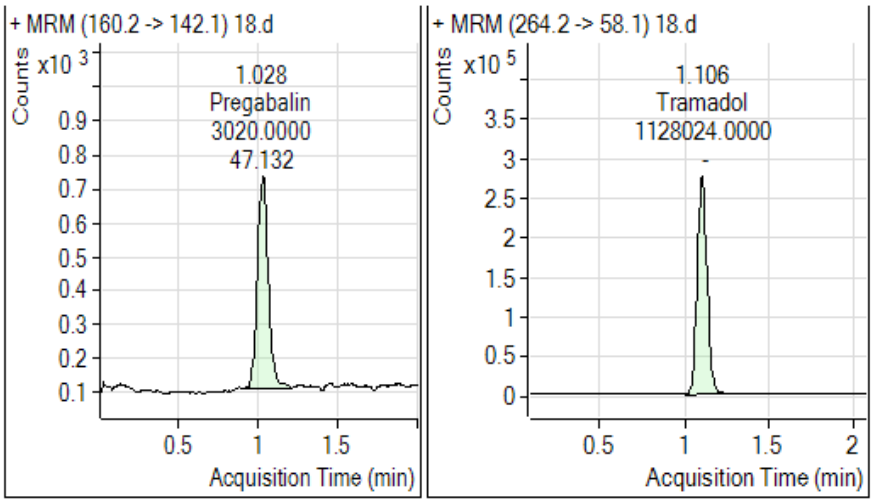

Fig. 4: Representative Chromatogram of Lower limit of quantification for pregabalin and Tramadol

Table 2: Concentration of pregabalin in calibration standards prepared in human plasma

	Nominal*	Mean	CV,%
A	50.000	49.450	1.82
B	100.000	98.120	4.70
C	375.000	376.240	1.39
D	750.000	749.249	1.97
E	2064.000	2062.670	0.45
F	4128.000	4127.972	2.45
G	6070.000	6072.366	1.45
H	8550.000	8549.974	2.68
I	10000.000	10012.465	1.72

*ng/ml; n = 4

Table 3: Data of Intraday and Inter day accuracy and precision

Intra day					Inter day		
Drug	**Nominal***	**Mean**	**Accuracy,%**	**CV, %**	**Mean**	**Accuracy,%**	**CV, %**
LLOQ, n = 6	50.00	54.53	109.060	6.766	55.71	111.42	2.307
LQC, n = 6	128.057	126.183	98.97	1.255	116.0533	90.62	0.141
MQC, n = 6	3201.419	34596.761	108.20	1.944	3461.616	108.12	0.985
HQC, n = 6	8003.548	8062.110	101.55	5.555	8580.414	107.207	1.714

***ng/ml**

Table 4: Concentrations of pregabalin in stability sample prepared in human plasma

Stability	Nominal*	% difference	CV, %
Freeze thaw	137.9985	- 2.00	2.52
	8160.017	- 4.72	4.35
Bench top	129.4592	3.56	7.61
	7662.997	1.28	6.77
Short term	137.3283	0.78	7.61
	8027.559	-1.07	6.77
Long term	128.057	4.26	1.80
	8003.548	2.52	1.81

*ng/ml n=6

CONCLUSION

The LC-MS/MS method described for pregabalin was simple, rapid, reproducible and suitable or their determination in human plasma. This method also has a good sensitivity, specificity and are also suitable for high throughput clinical sample analysis. There were no significant interferences and matrix effects by endogenous compounds throughout the analysis. These methods can also be used as therapeutic drug monitoring technique to evaluate the pharmacokinetic parameters of drug molecules in human plasma. The developed and validated method has its own advantage and significance which can be applied for successful clinical pharmacokinetic studies

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