Simple, rapid and highly sensitive HPLC method for measurement of Lamotrigine in human plasma and its clinical applications

Abdul Sami Shaikh[1,2], Wenlong Li[1], Guiyan Yuan[1], Meimei Gao[1], Chunmei Geng[1], Nan Guo[1] and Ruichen Guo[1*]

[1]Institute of Clinical Pharmacology, Qilu Hospital, Shandong University, Jinan, China
[2]Department of Pharmacy, Shah Abdul Latif University, Khairpur, Sindh, Pakistan

Abstract: Spikes in Lamotrigine concentrations levels and associated clinical toxicity may occur unpredictably. This study describes the development and validation of a simple, more rapid, highly sensitive and economical method for measuring Lamotrigine (LTG) concentration levels in human plasma using HPLC-UV and its clinical applications. Analyte from plasma was extracted with methanol (protein precipitation) and separated on the analytical column Diamonsil C_{18} (150mm×4.6mm, 5µm) Waters-Milford, MA, United States. Mixture of 0.1% Trifluoroacetate and Methanol used as mobile phase in a 59:41 volume/volume mixture with an isocratic flow rate of 1.5 ml/min and wavelength was adjusted to 260nm. Standard curve of lamotrigine showed good linearity over the range of 1.0-50µg/mL (r^2=0.9961) and LLOQ was 1.0µg/ml. The Specificity, Recovery, Accuracy, Stability, Robustness and RSDs for both intraday and interday precision were within acceptable limits. The highly sensitive HPLC assay for determination of LTG in human plasma was demonstrated, validated and applied in Therapeutic Drug Monitoring (TDM) of sixty seven epilepsy patients who were using LTG. The proposed method can be easily applied in routine Therapeutic monitoring of LTG, Besides TDM, stated method can be also very useful for Bioequivalence studies, Pharmacovigilance and Pharmacokinetics studies.

Keywords: Antiepileptic, lamotrigine, quantification of lamotrigine, TDM of lamotrigine.

INTRODUCTION

Lamotrigine (Brand Lamictal®) is a novel broad-spectrum antiepileptic drug (fig.1). It works by an inhibiting the voltage-sensitive sodium channels, and is thought to act by decreasing presynaptic release of glutamate (Geddes *et al*., 2016). A novel drug approved by FDA of US in late 1994 as an adjunctive therapy of partial seizures (Krasowski and McMillin, 2014) and also gained indication in bipolar disorder treatment (Krasowski, 2010). In terms of safety of pregnancy, Lamotrigine has a safe profile, which leads to it being commonly used in pregnant women (Sabers and Tomson, 2009). Lamotrigine is absorbed speedily and totally through the GI tract and only 50–60% binds with serum proteins. Lamotrigine also allocates into saliva, with an average approximately salivary concentrations 0.4–0.5 on that of serum concentrations in epilepsy patients getting lamotrigine treatment for long time. Lamotrigine's salivary concentrations are very well linked with those of serum, and this link marks saliva a substitute sample to accomplish Therapeutic Drug Monitoring TDM (Incecayir *et al*., 2007; Malone *et al*., 2006). The lamotrigine's metabolism is highly affected with the associated use of liver enzyme inducers. Lamotrigine when used as monotherapy, the serum half-life is typically 15–35 hours but when used concurrently with liver enzyme inducers, only 8–20 hours and up to 60 hours when consumed parallel with Sodium Valproate, a Cytochrome P450 (CYP) enzyme inhibitor (Biton, 2006). Ethinyl estradiol present in Oral contraceptives drugs notably decreases the lamotrigine's serum concentrations (Sabers *et al*., 2001). The lamotrigine's clearance is noticeably higher (~300%) during pregnancy and high in children (Perucca, 2006). There is no close correlation between clinical action and serum/plasma concentrations (Bartoli *et al*., 1997) but 3–14 mg/L as a standard reference range has been anticipated for refractory epilepsy therapy. When concentrations of lamotrigine in serum/plasma exceed 15 mg/L, the frequency of toxic effects is significantly high (Morris *et al*., 1998). Many studies published that LTG toxicity may be associated with higher concentration of LTG in plasma. Ramey P *et al* found that out of 922 patients with available levels, 22 epilepsy patients found at least one incident of Lamotrigine toxicity with concentration higher than 20 mg/l. The peak serum concentration/level varied from 21.1 to 40.3 mg/l with the mean of 28.7. The rise in level was explained in three patients (post-delivery in one patient, addition of valproate in two patients). In the others eighteen, the rise was not elucidated nor was it disproportionate to an increase in dose of lamotrigine. Sudden change in lamotrigine concentration levels and associated clinical toxicity may occur surprisingly, signifying that in some individuals, elimination kinetics may be nonlinear at serum concentrations in the upper limit. Development of new symptoms are warning signs

**Corresponding author:* e-mail: grc7636@126.com

for close monitoring and measurement of LTG levels which could be due to lamotrigine toxicity, especially if the baseline serum concentration has exceeds>10 mg/l. (Ramey *et al*., 2016). Special attention is required when LTG is administered with other antiepileptic drugs. In one study Takeuchi T *et al* reported that the concentration of LTG changes dramatically, when LTG is used with other antiepileptic drugs in Japanese children as earlier reported in other countries and matter needs special consideration. The adjustment in dose of LTG should be adjusted accordingly in starting or discontinuing of Sodium Valproate or metabolic inducers, while there is no need of adjustment if the changing of the dose of Sodium Valproate or metabolic inducers is in the therapeutic range (Takeuchi *et al*., 2016).

Numerous types of analytical approaches have been stated so for the measurement of lamotrigine in plasma/serum, which includes High Pressure Liquid Chromatography, Immunofluorometric assay (IFMA), Homogeneous immunoassay (EMIT), Capillary electrophoresis (CE), Radioimmunoassay (RIA), Capillary zone electrophoresis-electrospray ionization-mass spectrometry, Liquid Chromatography-Mass Spectrometry (LC-MS), Gas Chromatography-Mass Spectrometry (GC-MS), Gas chromatography (GC) with a nitrogen-phosphorus detector, Micellar electrokinetic capillary chromatography and Liquid Chromatography-Mass Spectrometry/ Mass Spectrometry (LC-MS/MS).

Our goal of this research study was to develop and validate a simple, more rapid, highly sensitive and cost-effective method/procedure for routine quantifying of Lamotrigine (LTG) concentration levels in human plasma using HPLC-UV.

MATERIALS AND METHODS

Chemicals

Lamotrigine (Code No. 100775-200401) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Chlorzoxazone (Internal standard, IS, Lot No. LQ20P10) was purchased through J&K chemical company. Chromatographic-grade Ethyl Acetate (Lot No.0000055440), Acetonitrile (Lot No. 0000059829) and Methanol (Lot No.0000066140) were kindly supplied by J. T BAKER Company. The water used was supplied by the Hangzhou Wahaha Group Co, Ltd China.

Instruments

The chromatographic system Waters HPLC (2695) furnished with Empower station was used of Waters Co, Limited, US. Analytical Electronic balances were used of Mettler Toledo Instruments (Shanghai) Co., Ltd China. Proind centrifuge machine was obtained through the Heraeus (Germany based company). Vortex Mixer model no (XW-80A) was obtained from Shanghai Jingke Industrial Co Limited China, and Sonic Washer model no (PK514BP) was acquired from BANDEL (Germany based) and thermostatic water tank model no (BHW-IV) in this study was used of Beijing Medical Equipment Factory.

Chromatographic conditions

Mobile phase comprised a blend of 0.1% trifluoroacetate and Methanol (59:41 v/v). The analytical column applied was Diamonsil C_{18} (150mm×4.6mm, 5μm Waters, Milford, MA, US). The column's temperature was adjusted at the 40°C. The applied flow rate was 1.5mL/min and 20μL of volume was injected in the HPLC system for analysis. The UV detection wavelength was maintained at 260nm.

Preparation of stock and working solution

(A) Lamotrigine stock solution. An accurately weighed 10.0 mg portion of LTG standard was moved to the 10 mL volumetric flask and methanol was added to dissolve the substance. The solution of the flask was further diluted with methanol to get a required volume. The resulting Lamotrigine concentration was 1000μg/mL. The working solution was diluted to 20, 50,100, 250, 500, 750 and 1000 μg/mL concentration levels with mobile phase for validation studies. All solutions were stable for 30 days when kept at 4°C and away from daylight.

(B) Chlorzoxazone stock solution. An accurately weighed 25.0 mg portion of chlorzoxazone standard was moved to a 25mL volumetric flask, methanol was added to dissolve the substance. The solution of the flask was further diluted with the methanol to make a concentration of 1000μg/mL chlorzoxazone. The final working solution was diluted to 300 μg/mL concentration level for validation studies. The stock and working solution were stable for 30 days when kept at 4°C and away from daylight

Blood sampling of patients

The study was approved by ethics committee of Shandong university china. Blood sampling of sixty seven epilepsy patients was carried out in Qilu Hospital, Shandong University, Jinan, China. After that patients sample tubes were processed for centrifuging at 5000rpm for 5 minutes at 25°C. The Plasma was moved in the Eppendorf tubes for further analysis. Patient plasma samples were kept at –20°C before measurement.

Sample preparation

All the samples including Calibration and Quality Control (QC) samples were arranged throughout the process of method validation and determination of plasma samples. The concentrations of the calibration plasma samples were 1.0, 2.5, 5.0, 12.5, 25.0, 37.5 and 50.0μg/mL respectively. (200 μL) of patient plasma mixed with 10

µL of internal standard chlorzoxazone, vortexed for 1 min, 460 µL of methanol added and vortexed again for 2 min and centrifuged at 10800 rpm for 10 min for getting clear solution and then supernatant 20µL was inserted in the HPLC system for the measurement or analysis.

Method validation

Simple, rapid, accurate, highly sensitive and inexpensive High-Pressure Liquid Chromatographic (HPLC) technique employing the UV detection for determination of Lamotrigine in the human plasma has been established, recognized, tested and validated with the standard guidelines of FDA for routine use in applications of TDM, Pharmacovigilance, Bioequivalence studies and Pharmacokinetic studies. The following parameters were observed.

Specificity: The proposed method's specificity was appraised by comparing the chromatograms of: A; Lamotrigine and IS standard solution, B; Blank Plasma, C; Lamotrigine concentration of 1.0ug/ml in blank plasma spiked with IS (LLOQ), D; Patient plasma after administration of Lamotrigine spiked with IS.

Recovery: Peak areas of Standard solutions of Lamotrigine or IS, to the peak areas of plasma samples to which Lamotrigine or IS has been added were compared for the calculation of extraction recoveries.

Calibration curve and the lowest limit of quantitation (LLOQ): The concentrations of the calibration curve were 1.0, 2.5, 5.0, 12.5, 25.0, 37.5 and 50.0µg/mL in the plasma. The calibration samples of each concentration were analyzed in five replicates. Calibration curve was calculated by plotting the peak area ratios of Lamotrigine to IS chlorzoxazone. The lower limit of quantitation (LLOQ) was 1.0µg/ml concentration, examined by analyzing five replicates of mixed plasma samples. The standard calibration curve was plotted by analyzing the seven dissimilar concentrations of calibration plasma samples on each separate day of analysis which is typically explained by the equation ($y = ax + b$), where y is representing to the peak-area ratio and x represents to the concentration ratio of Lamotrigine to IS, and the linearity of standard calibration curve was evaluated via linear regression with the weighting factor of the reciprocal of the concentration squared ($1/x^2$).

Accuracy and precision: Each sample of LQC (2.5 µg/ml), MQC (12.5 µg/ml) HQC (40µg/ml) concentration processed five times for calculating the Intra-day accuracy and precision. The Inter-day precision of each assay was analyzed built on five same concentrations of each LQC, MQC and HQC in three dissimilar days over the period of one week. Similar data was used for calculation of Accuracy and precision. Calibration curves were constructed using the concentrations of Lamotrigine against peak area ratios (a peak area of LTG over peak area of IS). Precision is expressed as the relative standard variation (RSD).

Stability: The stability of LTG in samples was evaluated and the final results are stated in percentage (%) recoveries. The stability of Lamotrigine in plasma samples at various different concentrations was evaluated below various numerous study conditions, such as storing the samples at -20°C for 60 days. The freeze–thaw stability was analyzed after the freezing the samples at (-20°C) and thawing at (25°C) for two cycles. The stability of the post-extracted samples on the bench top and within the HPLC-UV auto-sampler was also observed for 8h and 10h at room temperature.

Robustness: Robustness of the stated method was determined by deliberately changing the flow rate (1.4–1.6 mL/min) and column temperature (35-45°C) and change in the percentage of mobile phase (57:43) and change in percentage of Trifluoroacetate in water (0.05-0.15%).

RESULTS

Specificity: The typical chromatograms of Lamotrigine and IS are drawn below in (fig. 2). There were no any noteworthy interference seen in the endogenous blank human plasma at retention time of the Lamotrigine and IS.

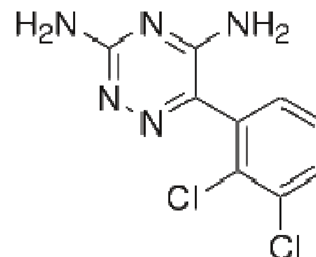

Fig. 1: Chemical structure of Lamotrigine.

Recovery: A simple protein precipitation method has proved to be vital and method provided the clean samples. The recoveries were greater than 83% for Lamotrigine and 101% for IS, which are shown in table 1.

Calibration curve and Lowest limit of the quantitation: The standard calibration curve of Lamotrigine was linear within the concentration range of (1.0-50µg/mL) with Regression equation: (weight=$1/X^2$), Y=3.89e+000X+1.40e+000 (r^2 =0.9961) (fig. 3). The LLOQ of Lamotrigine (1.0µg/mL) was sensitive enough.

Accuracy and precision: All the results of Accuracy and precision for analysis of Lamotrigine are displayed in table 2. The RSDs for both intra-assay and inter-assay precision were below 4% for the entire quality control samples. And the accuracy was ranged from 100.3% to 103%.

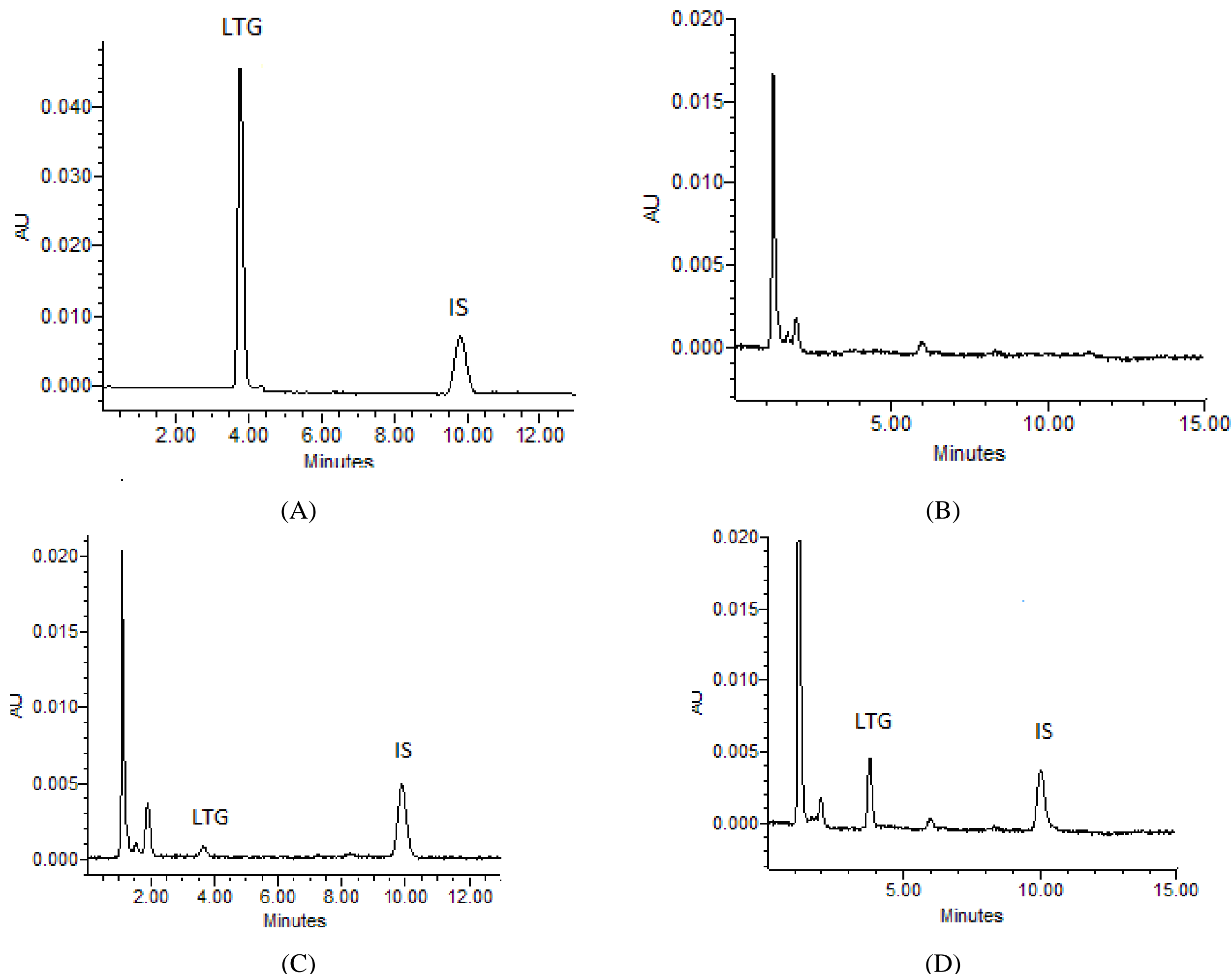

Fig. 2: The typical chromatograms of Lamotrigine and Internal Standard (IS) chlorzoxazone A; Lamotrigine and IS standard solution, B; Blank Plasma, C; Lamotrigine concentration of 1.0ug/ml in blank plasma spiked with IS (LLOQ), D; Patient plasma after administration of Lamotrigine spiked with IS.

Stability: The stability of Lamotrigine in plasma was inspected under various numerous storage conditions and processing. Lamotrigine was stable after 8 hour on bench-top, 10 hour in auto sampler, two freeze–thaw cycles and frozen at -20°C for fourteen days. The results are available in table 3.

Robustness: No significant changes were observed by deliberately changing the chromatographic conditions which endorses the robustness of the proposed method.

Application in TDM: We proved the suitability of our method for Therapeutic Drug Monitoring (TDM) by testing the plasma samples of sixty seven epilepsy patients who were using LTG. Measured range of lamotrigine concentration in all sixty seven plasma samples was between the 1.38- 12.49 mg/L, while range of 3–14 mg/L as a standard reference range of Lamotrigine has been anticipated for refractory epilepsy therapy. The Minimum Effective Concentration (MEC) is 3mg/L, but we found that some plasma samples were having concentration of less than 3mg/L. So it was indicating that further necessary measures should be carried out to find out the route cause and adjusting the dosage regimens accordingly for these individuals. Stated method is simple, more rapid, reliable, sensitive, and cost-effective. The proposed HPLC-UV method is suitable for TDM of lamotrigine. Besides that, the proposed method can be also easily applied to bioequivalence studies that require the comparisons of brand versus generic lamotrigine; method is also helpful for monitoring and improving the Pharmacovigilance and Pharmacokinetics studies.

DISCUSSION

In this study, Simple, rapid, accurate, sensitive and inexpensive HPLC method for determination of the Lamotrigine in human plasma has been developed and validated in our laboratory with the above described validation strategies. The statistical validated method displays a decent intra-assay and interassay precision (less

Table 1: Results of recovery (n=5)

	C(ug/mL)	Mean±SD	RSD (%)
LTG	2.5	84.81±2.08	2.4
	12.5	85.74±1.21	1.4
	40	83.49±0.4	0.5
IS	15	101.14±4.27	4.22

Table 2: Results of accuracy and precision (n=5)

	Inter-day			Intra-day		
C (ug/mL)	Mean±SD	Accuracy (%)	RSD (%)	Mean±SD	Accuracy (%)	RSD (%)
2.5	2.53±0.01	101.2	0.57	2.50±0.02	100	0.80
12.5	12.46±0.2	99.7	1.60	12.51±0.072	100.0	0.58
40	41.44±1.45	103.6	3.49	40.64±1.26	101.6	3.10

Table 3: Results of stability

C(µg/mL)		Bench top (8 h)	auto sampler (10 h)	Two freeze-thaw cycles	14 days at -20 °C
2.5	Mean ± SD	2.55±0.14	2.43±0.05	2.42±0.27	2.61±0.21
	Accuracy (%)	102	97.2	96.8	104.4
12.5	Mean ± SD	12.46±0.11	12.31±0.21	12.37±0.48	12.98±0.51
	Accuracy (%)	99.7	98.5	98.9	103.8
40	Mean ± SD	40.44±1.02	41.4±0.7	39.68±0.93	39.14±0.41
	Accuracy (%)	101.1	102.6	99.2	97.8

than 4%) and accuracy (99.7% to 103.6%) in the entire concentration range and having ideal extraction efficiency/recovery (more than 83%). The quantitation range of method is satisfactory for Lamotrigine purposes even the patients getting very low daily dosages, as the LLOQ of the method is at lowest concentration of 1.0µg/ml. The run time of chromatographic analysis for each sample was only 15 min.

The described method showed to have acceptable adoptability, specificity, sensitivity, robustness, accuracy and precision for routine analysis of the Lamotrigine in patients of epilepsy. Utilization of the proposed method was practiced to the quantification of LTG in sixty seven epilepsy patients referred to Qilu hospital treated with LTG and results were revealing that there is need of further evaluation and adjusting the dosage regimens in individual patients is necessary for getting positive results. Various neurologists, psychiatrists and epilepsy patients raised worries regarding the bioequivalence of brand Lamictal to the generic lamotrigine. Requirement of Bioequivalence is also the quantification of lamotrigine alone or concomitant therapy of Lamotrigine with other drugs in human plasma of epilepsy patients rather than healthy volunteers (Wong *et al.*, 2015). The focuses of Pharmacovigilance are to supervise and prevent any drug-related problems under everyday circumstances. Pharmacovigilance is one of the main suggestions for evaluating plasma/serum concentrations, i.e. conducting therapeutic drug monitoring (TDM). By using therapeutic drug monitoring, it can be explained very well if perceived unwanted drug effects may be accredited to abnormally high or low drug concentrations. Highest benefits through TDM can be gained for monitoring of Pharmacovigilance especially if the technique is sufficiently incorporated into the process of clinical treatment. To the best of our knowledge, the proposed method has highly sensitive LLOQ (1.00 ug/mL) over earlier reported methods by HPLC, besides that proposed method is simple, very fast, economical and having shorter runtime. This method also has nominal sample pretreatment which permits a huge sequences of patient samples can be run in a very little time; which is a very advantageous job in a Therapeutic Drug Monitoring (TDM) setting. Besides that, the simple reversed-phased RP-HPLC chromatographic technique is simple technique which has the advantage of adoptability even in simple laboratories which are lacking sophisticated analytical instruments.

CONCLUSION

A simple, sensitive, reliable, rapid and inexpensive HPLC-UV method for the routine analysis/ determination of Lamotrigine in the human plasma is designated and furthermore suitability of method for TDM was confirmed by testing of plasma of sixty seven epilepsy patients and results were stating that initiating of

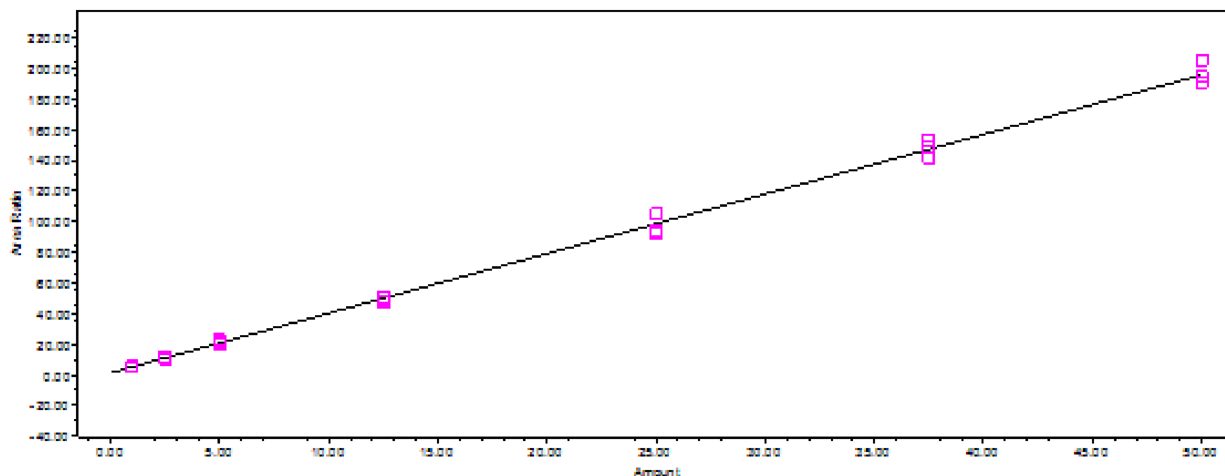

Fig. 3: Standard curve of lamotrigine (1.0-50µg/mL).

necessary measures to find out route cause and adjustments in dosage regimens are very essential for better outcomes and avoiding unnecessary complications. Potential benefits of proved method can be easily gained in clinical practice. Stated method is very helpful and guide for physicians and other health professionals in clinical practice for getting better results. The method has an appropriately speedy turnaround time, reproducible and the results are satisfactory ample and within the acceptable range to permit the laboratory to routinely provide valuable and accurate TDM and pharmacokinetic statistics in very short time to adjust the patient dose regimens. The proposed HPLC-UV method has proved to be useful for TDM, and can be also useful for Pharmacovigilance, Bioequivalence, Pharmacokinetics studies and for evaluating the toxic effects associated with unexplained change in lamotrigine concentration.

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