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A Validated Bioanalytical Method for Simultaneous Analysis of Lamotrigine, Oxcarbazepine and Levetiracetam by High Performance Thin Layer Chromatography

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KEYWORDS

Epilepsy, Simultaneous estimation, Lamotrigine (LTG), Oxcarbazepine (OXC) and Levetiracetam (LVT)

ABSTRACT:

Simultaneous analysis by chromatography is utilized for quantitative estimation of antiepileptic drugs in combination therapy. This research paper portrays a rapid HPTLC method for simultaneous analysis of Lamotrigine (LTG), Oxcarbazepine (OXC) and Levetiracetam (LVT) in the mobile phase Dichloromethane: Methanol and quantitated at 210nm to give a retention time of 0.17, 0.26, 0.48 respectively. Linearity curve was established in the range of 500-3000 ng/band for all the three drugs. Validation as per ICH guidelines showed that the method was specific, accurate, robust and reproducible for simultaneous estimation with the %RSD value less than 2%. Specificity was evaluated by an assay of marketed formulation and it gave a percent content between 95.71-102.52%w/w for all the three drugs. The validated HPTLC method was used for analysis of drugs extracted from plasma. Linearity, precision, accuracy and matrix effect was studied on drug samples extracted from plasma by the protein precipitation method. Human plasma stability experiments were undertaken to detect degradation of drugs under specified conditions of stock solution stability, freeze thaw stability and bench top stability.

1. Introduction

Epilepsy , a neurological disorder manifests as abnormal brain activity disorder, causing seizures or episodes of unusual behaviour, sensations and occasional loss of awareness. The disorder encompasses of mainly three types monoclinic seizures, absence seizures, and generalized tonic clonic seizures[1] and to cure them we have two approaches : monotherapy and polytherapy. Polytherapy becomes a viable option as it involves the simultaneous administration of two or more drugs. The antiepileptic drugs (AEDs) market has seen the introduction of many new drugs characterized by improved tolerability and reduced potential for drug–drug interactions, and advancement in their pharmacokinetics[2]. Three second generation AEDs namely, Levetiracetam (LVT), Lamotrigine (LTG) and Oxcarbazepine (OXC) were chosen for the study. The drug Levetiracetam [(S)-2-(2-oxopyrrolidin-1-yl)butanamide] a broad spectrum AEDs [3] is specified as an adjunctive therapy for partial onset seizures in epileptic patients of one month of age and older. It is also given in myoclonic seizures in juvenile patients (12 years and above) and in primary generalized tonic-clonic seizures in patients with idiopathic generalized epilepsy [4]. Lamotrigine [6-(2,3-Dichlorophenyl)-1,2,4-triazine-3,5-diamine] [5] is used for treating both epilepsy and as a mood stabilizer in bipolar disorder. It is an organic cation transporter 2 inhibitor and dihyrofolate reductase inhibitor [6]. Oxcarbazepine [7] (5-oxo-6H-benzo[b][1]benzazepine-11-carboxamide) is used for both focal seizures and generalized seizures. Oxcarbazepine, a pro-drug in humans, is completely absorbed by the gastrointestinal tract by blocking voltage-sensitive sodium channels [8]. Literature reports variety of HPLC techniques for assessment of antiepileptic drugs in bulk and plasma samples [9-10]. Simultaneous quantitation of four antiepileptic drugs Lamotrigine (LTG), Levetiracetam (LVT), Phenobarbital (PBT) and Phenytoin (PTY) was established in our laboratory using C18 column in JASCO HPLC in the mobile phase 5 mM potassium

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phosphate buffer (pH 6) and acetonitrile at a flow rate of 1ml/min and detection done at 230 nm using UV detector [11]. Analysis of lamotrigine using methanol :10 mM, ammonium acetate (pH 3.0 ± 0.2 adjusted with formic acid) 30:70 (v/v) as mobile phase with a flow rate of 1.0 ml/min is reported [12]. Quantitation of Levetiracetam in tablet and urine sample described by Tyagi [13] consisted of Methanol: Water: Triethanol amine 70:25:05 (v/v) with detection at 224 nm. There are methods available for the selected drugs using LC-MS and HPLC; however use of HPTLC analysis was restricted for single drug or for combination of two in pharmaceutical dosage forms [14-15].

The foremost advantage of HPTLC is that a number of samples can be developed concurrently using less quantity of mobile phase, thus lowering the analysis time and cost. Literature search reveals that levetiracetam was quantitated at 210 nm using Toluene: Acetone: Methanol (6:2:2) v/v) on Silica Gel 60 F254 plates [15]. Separation of the drug lamotrigine from its forced degradation impurities was established using TLC silica gel plates and mobile phase composing of ethyl acetate: methanol: ammonia [16]. Appraisal of the greenness profile of a chromatographic method for the simultaneous estimation of carbamazepine and oxcarbazepine, along with impurities and formulation excipients using a mixture of hexane : ethylacetate : formic acid : acetic acid (8 : 2 : 0.5 : 0.3) by UV 254 nm was reported [17].
Simultaneous estimation of drugs would help in therapeutic drug monitoring when patient is given combination therapy. Literature reports the quantitative estimation of selected antiepileptic agents individually in pharmaceutical formulation and in biological fluids using HPLC-MS/ UPLC [18,19] however, there were no methods available for simultaneous estimation of all three drugs together using simple analytical techniques like HPTLC. The aim hence was simultaneous quantitation of three antiepileptic drugs Levetiracetam, Lamotrigine, and Oxcarbamazepine using HPTLC method. The validated HPTLC method was further applied for analysis of drugs extracted from plasma (*in vitro* studies) which can be further extrapolated to *in vivo* bioanalytical studies.

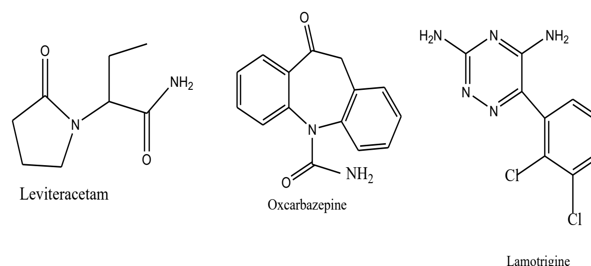

Figure. 1. Chemical structures of lamotrigine, Oxcarbazepine and Levetiracetam.

2. Materials and method:

Material:
Lamotrigine (assay ≥ 98%) was obtained as gift sample from CTX life sciences, Surat, Oxcarbazepine (assay ≥ 98%) from Amoli organics Pvt.Ltd. and Levetiracetam (assay ≥ 98%) from Abbott laboratories, Mumbai. The purity of the APIs was approved by melting point and spectral analysis in the UV-1900 Shimadzu. Methanol used was of HPLC grade and DCM was of analytical grade, and both the chemicals purchased from Merck (Darmstadt, Germany). The marketed tablets Laminext 25 DT (Lamotrigine dispersible tablets 25 mg), Lysiprin 250 (Levetiracetam tablets IP 250 mg), Oxacent 300 (Oxcarbazepine tablets IP 300 mg) were used for accuracy studies.

Instrumentation and Chromatographic conditions:
HPTLC was achieved on 10 cm × 10 cm aluminum-backed silica gel 60 GF254 plates, (20-μm thickness, E. Merck, Germany) with a Camag Linomat V (Switzerland). Sample bands of 6-mm was applied by use of a Camag (Switzerland) Linomat V sample applicator using a microliter syringe. Nitrogen aspirator was used to obtain an application rate of 150 nL s^{-1}. The plates were scanned using Camag TLC scanner 3 and winCATS software 1.4.4.6337 with a slit dimension of 5 × 0.45 mm and 200 mm/s scan speed. The source of radiation was deuterium lamp with a wavelength of 210 nm for all the three test samples. Different combinations of mobile phase using dichloromethane, methanol, toluene, hexane, ethyl acetate, and formic acid were tried. The best resolution was obtained using dichloromethane and methanol (9.5:0.5). A chamber saturation time of 20 minutes and the migration distance of 75 mm was maintained in a twin trough chamber.

Analytical Method Development and Validation:

Preparation of stock solution:

A 1000µg/ml stock solution of each drug was prepared in methanol. 5 ml of stock solution was diluted to 50 ml to get concentration of 100µg/ml

Preparation of Sample solution:

10 tablets of each drug were weighed, average weight determined and the tablets triturated to get fine powder. As per label claim, powder equivalent to 50 mg of drug was weighed and transferred to 100 ml volumetric flask. The solution was filtered and the resulting solution gave a 500 ppm concentration.

Method validation:

Calibration solutions were freshly prepared from 100µg/ml solutions of Levetiracetam, Lamotrigine, Oxcarbazepine (LVT, LTG, OXC). A calibration curve of concentration v/s absorbance was plotted, and linearity established in the range of 500-3000 ng/band for all the three drugs. The method validation was carried out according to ICH guideline Q2(R1) [20,21] for precision, specificity, accuracy, limits of detection (LOD), limits of quantification (LOQ) and robustness. Specificity was ascertained by analyzing individual standard drugs and mixture of all drugs as a sample and the results compared with the chromatograms of marketed formulations. Assay of the marketed formulations was performed, and drug content calculated from the linear regression data. Accuracy was determined by the percent recovery method on the marketed tablets. The sample solution 500 ppm was prepared in methanol and was spiked with individual standard drugs at different levels i.e., 50%, 100%, 150%, thus making the overall concentration of the stock solutions at three different levels as 750 ppm, 1000 ppm and 1250 ppm. A 10 times dilution was carried out to get a concentration in the linearity curve, which was detected by the proposed method. For the evaluation of precision, intra-day and inter day variances were determined over a period of 1 and 3 days, respectively. In the intra-day studies, 3 replicates of standard solutions (1000, 1500 and 2000 ng/band) were analyzed in a day and %RSD was determined. For inter-day precision studies, 3 replicates of standard solutions (1000, 1500 and 2000 ng/spot) were analyzed in three consecutive days and %RSD was calculated. Precision studies were carried out for each drug under study. The sensitivity for the simultaneous determination of the three antiepileptic drugs was evaluated with respect to the limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were calculated as per the formula given in the ICH guidelines from the data derived from the calibration curve of each standard. The robustness of the method was studied, by small but deliberate variations in the chromatographic conditions, i.e. time of development of scanning (scanning was executed soon after drying of the plate, after a few hours of development, next day), and change in mobile phase composition.

Bioanalytical method development and validation:

Bioanalytical method was developed using plasma as a biological matrix [22]. Plasma was obtained from Plasma blood bank, Dombivli (E) and stored in a deep freezer at -18°C when not in use.

Preparation of standard stock solution

A 1000 ppm solution of individual drugs (LVT, LTG, OXC) was prepared in methanol. 1 ml of standard stock solution (1000µg/ml) was spiked into 1 ml of plasma, it was then extracted by protein precipitation solvent extraction method, diluted 10times with methanol to get a working concentration of 100µg/ml for individual drugs. Similarly, for combination, 1 ml of each standard stock solution was added to the plasma and treated as mentioned above. Concentration of each drug in the mixture was 100µg/ml.

Protein precipitation method : In this method 1 ml of stock solution (1000 ppm) and 1 ml plasma were added into the centrifuge tube, vortex mixed for 5 minutes. 1 ml of Methanol was added and further centrifuged at 4000 rpm for 15 minutes resulting in protein precipitation. The supernatant was filtered and re-centrifuged in the same conditions and filtered in a 10ml volumetric flask. The volume was made to 10 ml using methanol to give a concentration of 100µg/ml.

Method Validation

The matrix effect was evaluated by analyzing 100µg/ml. working standard solution prepared as mentioned above using the protein precipitation method. 10µl band (1000 ng/band concentration) was spotted and developed in the optimized mobile phase and the effect of matrix was evaluated based on the analyte response. The developed analytical method was also validated as per ICH guideline M 10 for bioanalytical studies [23]. Linearity, precision, LOD and LOQ, were performed similar to analytical method validation in the same concentrations

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but by using drug samples extracted from plasma. For HPTLC studies 1ml of stock solution 1000ppm was added into a centrifuge tube containing 1ml of plasma. Protein precipitation and extraction was carried out by using methanol at 4000 rpm for 15 minutes. The organic phase was recovered and volume made upto 10ml using methanol to give a concentration of 100 µg/ml. Specificity was determined by analyzing blank plasma and spiked plasma for the absence of any interference at the Rf of analytes. Blank plasma sample was prepared by adding 1 ml of Methanol to 1 ml of plasma in a centrifuge tube and treated and scanned at the wavelength of 210nm. Absolute recovery was calculated by comparing the areas of spiked plasma samples with the samples prepared using only methanol at three levels of concentration similar to precision studies

Stability studies : Human plasma stability experiments were undertaken to detect degradation of drugs under specified conditions. In stock solution stability, the standards were stored in the specified solvent and checked at room temperature for 6hrs and at 4 °C for 30 days. Freeze–thaw stability in plasma was determined after freezing (−20 °C) for 24 hrs and thawing at room temperature. Bench-top stability in plasma was assessed by comparing the concentration of drug stored at room temperature for 6 hours with the concentration of drug obtained immediately after spiking the drug

3. Results

Analytical Method development and validation : The drugs Lamotrigine, Levetiracetam and Oxcarbazepine are soluble in methanol. HPTLC method was established in the mobile phase comprising of Dichloromethane: Methanol (9.5:0.5) and detection at 210 nm. The mean retention factor for LTG, LVT and OXC were found to be 0.17, 0.26, 0.48 respectively as seen in fig no. 2. The calibration curve was found to be linear in the range of 500ng/band to 3000ng/band with an R^2 value between 0.997 to 0.999. The LOD and LOQ was found to be 146.05ng/band and 442.56ng/band for levetiracetam, 80.46 and 243.84 ng/band for lamotrigine, 150.71ng/band and 456.70ng/band for oxcarbazepine respectively. The specificity of the method ascertained that there was no difference between Rf of sample and standard. Further it was observed that there was no interference seen in the analyte peak of marketed sample. The results of the assay of marketed sample was 102.52±2.01, 95.71±1.09 and 97.95±1.92 Lamotrigine, Oxcarbazepine and Levetiracetam respectively

The precision of the proposed method was checked by intra-day and inter-day repeatability of response and the %RSD values was found to be <2.0 %, thus stating that the method is precise for the drugs in the given range (Table 1).. The accuracy of the proposed method was determined by recovery studies at three different levels, i.e.50%, 100%, and 150%. All the accuracy values were within 85–115% of the recovery range as seen in Table no.2 suggesting that the described method was accurate. All experiments in robustness testing were performed in a randomized fashion to minimize the effects of uncontrolled factors that may introduce bias to the response. When the mobile phase composition was altered, the retention factor changed because methanol content in the mobile phase had the most important effect, however the percent RSD was less than 2%. The other examined factors, development time and scanning time had no significant effect on retention factor and area. The method passed all the acceptance criteria and was found to be robust as seen in Table no. 3

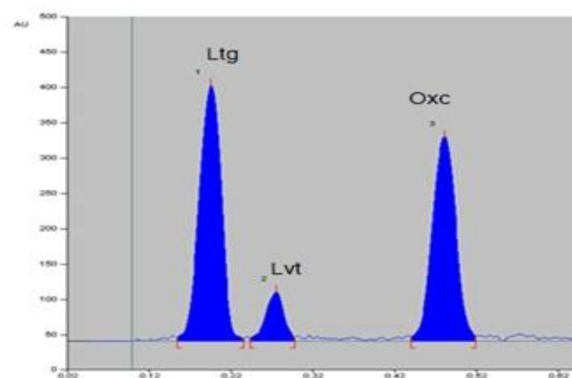

Figure no. 2: **HPTLC densitogram of Lamotrigine, Levetiracetam & Oxcarbazepine (Wavelength: 210 nm)**

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Table no. 1 Precision Studies for the Analytical Method

Analyte name	Concentration level	Intraday precision Mean area ±SD	%RSD	Interday precision Mean area ±SD	%RSD
Lamotrigine	LQC	6556.4± 125.95	1.92	6561.68±112.67	1.72
	MQC	8746.05±172.05	1.96	8701.90±161.77	1.86
	HQC	11518.65±190.34	1.65	11473.74±209.08	1.82
Oxcarbazepine	LQC	6279.57±47.44	0.75	6337.23±99.02	1.56
	MQC	7953.38±38.57	0.48	7884.78±108.18	1.37
	HQC	9098.78±176.02	1.93	9158.8±169.15	1.84
Levetiracetam	LQC	1285.15±17.45	1.35	1301.23±14.04	1.07
	MQC	1512.15±25.57	1.69	1521.94±18.37	1.21
	HQC	1808.30±7.33	0.40	1808.87±9.52	0.52

Table no. 2 Accuracy results or recovery studies of developed analytical Method

Sample	Sample amount taken (µg)	Standard amount Spiked (%)	Standard amount Spiked (µg)	Total Amount Taken (µg)	Total Amount found (mg) (mean±SD)	% Recovery (mean±SD)
Lamotrigine	50	50	25	75	74.76±0.51	99.67±0.67
		100	50	100	98.04±4.72	100.04±1.96
		150	75	125	124.89±3.77	101.25±0.96
Levetiracetam	50	50	25	75	71.49±1.61	95.66±1.67
		100	50	100	98.25±1.14	98.25±1.14
		150	75	125	123.89±0.76	99.12±0.63
Oxcarbazepine	50	50	25	75	76.02±1.03	101.37±1.38
		100	50	100	101.29±1.24	101.29±1.24
		150	75	125	125.35±2.54	99.95±1.51

Table no. 3 Robustness studies .

Parameter		%RSD for peak area			Rf value		
		Ltg	Oxc	Lvt	Ltg	Oxc	Lvt
Development time – scanning time	**2 hours**	0.42	0.58	0.92	0.18	0.49	0.26
	24 hours	0.56	0.87	0.95	0.17	0.48	0.25
Mobile phase composition	**DCM: MeOH (8:2)**	0.43	0.86	0.90	0.35	0.72	0.48
	DCM: MeOH (9:1)	0.56	0.94	0.98	0.22	0.54	0.31

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Bioanalytical Method Development and Validation: Bioanalytical method was developed using plasma as a biological matrix. Plasma obtained from Plasma blood bank; Dombivli was stored in deep freezer at -18°C when not in use. Protein precipitation method was used for the extraction of drugs from the plasma. Methanol was found to be the most suitable solvent for extraction of the drugs. It was observed that due to matrix effect shown by the plasma, there was a change in the Rf of Lamotrigine from 0.17 to 0.21 and Levetiracetam from 0.22 from 0.26.

The developed method was validated as per ICH guideline M 10. Linear relationship between peak area and concentration of individual drugs was evaluated by spiking known amount of standard to the plasma and recovering the same using protein precipitation method. The method was found to be linear in the given range with R^2> 0.995 for all the three drugs. The LOD and LOQ were calculated as per the specified formula in the ICH guidelines and was found to be 154.76ng/band and 481.09ng/band for levetiracetam, 74.20 and 224.80 ng/band for lamotrigine, 182.73ng/band and 417.27ng/band for oxcarbazepine respectively. The interday and intraday precision of the proposed bioanalytical method was calculated and %RSD was found to be n.m.t.2.0 %; thus, stating that the method is precise for the drugs in the given range as seen in Table 5. The accuracy was evaluated and was expressed as % recovery between the concentration established and the concentration added for all the drugs. The recovery obtained by protein precipitation method was found to be less and not within the acceptable range. Sophisticated extraction techniques like solid phase micro-extraction can be used to get higher recovery. The stability experiments were conducted at three experimental conditions: 1. Stock solution stability (Room Temperature 6hrs and refrigerated at 4ºC) 2. Bench top stability (Comparison of the nominal value and plasma stored at Room temp for 6hrs) 3. Freeze thaw stability (After freezing at -20ºC for 4hrs and thawing at room temperatures). Above experimental conditions were tested at two levels (LQC, HQC). The results are represented in Table 6 and the %RSD was <2%

Table no. 4 Precision studies for Bioanalytical Method at three level:

Analyte name	Concentration (ng/band)	Intraday precision		Interday precision	
		Mean area ±SD	%RSD	Mean area ±SD	%RSD
Lamotrigine	1000	5663.28±29.03	0.52	5668.72±26.95	0.47
	1500	7805.33±43.29	0.55	7810.25±36.37	0.46
	2000	9356.15±85.89	0.92	9391.97±88.63	0.94
Oxcarbazepine	1000	3664.76±31.42	0.86	3687.53±42.64	1.15
	1500	5542.43±52.47	0.94	5560.21±49.69	0.89
	2000	7711.56±88.63	1.14	7731.4±78.19	1.01
Levetiracetam	1000	804.38±7.16	0.89	799.7±7.49	0.93
	1500	1074.4±12.36	1.15	1067.05±9.63	0.90
	2000	1364.21±14.69	1.07	1363.54±15.07	1.11

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Table no. 5: Results of accuracy studies Bioanalytical Method development:

Analyte	Concentration	Mean area of unextracted sample	Mean area of extracted sample	%Recovery	Mean±SD	%RSD
Lamotrigine	LQC	6516.26	5673.56	87.06	83.96±4.8	5.71
	MQC	8619.8	7392.96	85.76		
	HQC	11453.7	8993.33	78.52		
Oxcarbazepine	LQC	6294.96	3639.93	57.82	70.01±12.4	14.77
	MQC	7949.2	5526.46	69.52		
	HQC	9257.73	7656.5	82.70		
Levetiracetam	LQC	1292.83	799.06	61.80	69.26±7.04	10.16
	MQC	1519.16	1066.53	70.20		
	HQC	1808.86	1370.8	75.78		

Table 6: Stability studies for bioanalytical method validation

Stability para meters	Conce ntration	Lamotrigine			Leviteracetam			Oxcarbazepine		
		Mean Peak Area	% Concent ration	% RSD	Mean Peak Area	% Conc.	% RSD	Mean Peak Area	% Conc.	% RSD
Stability at RT	LQC	5574.2	98.43	1.13	794.12	98.72	1.2	3518.21	96.0	0.76
	HQC	8947.14	95.62	0.96	1341.68	98.34	0.96	7642.51	99.1	0.84
Refrigerator at 4°C	LQC	4784.70	84.48	1.78	686.21	85.30	1.35	3485.15	95.07	1.12
	HQC	7692.47	82.21	1.49	1209.30	88.64	1.1	7612.67	98.71	1.24
Freeze–thaw	LQC	5146.9	90.88	0.89	713.81	88.74	1.12	3249.45	88.66	0.54
	HQC	8617.53	92.15	0.75	1214.35	89.01	1.74	7549.70	97.9	0.62
Benchtop Stability	LQC	5617.62	99.19	0.93	790.71	98.30	0.85	3596.23	98.13	0.96
	HQC	9012.48	96.32	1.26	1304.24	95.6	0.87	7613.51	98.72	0.73

Conclusion:

A HPTLC method was developed for simultaneous determination of Lamotrigine, Oxcarbazepine and Levetiracetam and the method was validated. Mobile phase used in the developed method was DCM: Methanol (9.5:0.5) at a wavelength of 210 nm. The developed analytical method was found to be novel, simple, accurate, precise, specific, and reproducible for the simultaneous estimation of Lamotrigine, Oxcarbazepine and Levetiracetam. The method was further extrapolated to *in vitro* bioanalytical studies and gave good results .

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