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Article

Simultaneous Estimation and Validation of Four Antiepileptic Drugs from Bulk and Formulations Using Reverse Phase HPLC

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Epilepsy is a disorder of the central nervous system, in which the nerve cell activity in the brain is disturbed causing seizures. The objective was to develop an RP-HPLC method for consistent simultaneous quantitation of four antiepileptic drugs Levetiracetam (LVT), Lamotrigine (LTG), Phenobarbital (PBT) and Phenytoin (PTY). An isocratic method was developed on C18 column in JASCO HPLC using 5 mM potassium phosphate buffer (pH 6) and acetonitrile as the mobile phase at a flow rate of 1ml/min and detected at 230 nm using UV detector. The mean retention time for LVT, LTG, PBT and PTY were found as 2.55, 3.55, 4.65 and 5.99 minutes respectively. The method was validated as per ICH guidelines and was found to be acceptable. The %RSD value was <2.0 % thus stating the developed method was precise for the drugs in the given range. The accuracy values were within 85–115% of the recovery range. The specificity of the method was evaluated by an assay of marketed formulation, and it showed a percent content between 90–110% w/w for all the four drugs. The proposed analytical method was simple, accurate and robust and was precisely able to resolve the four major antiepileptic drugs. Hence, the current method can be applied successfully for routine examination of these drugs.

KEYWORDS: Antiepileptic. HPLC. Simultaneous estimation. Validation.

INTRODUCTION

Epilepsy is a chronic disorder in which brain activity becomes abnormal, causing unprovoked, recurrent seizures or periods of unusual behavior, sensations and sometimes loss of awareness. Epilepsy may occur due to a genetic disorder or an acquired brain injury, such as trauma or stroke. There are basically two approaches to cure epilepsy, namely monotherapy and polytherapy. Usually, the treatment starts with monotherapy i.e., a single drug molecule. Monotherapy is associated with minimum side effects, so it is preferred, but it does not always cure the disease. The goal of the therapy is to completely control seizures without producing unacceptable medication side effects. In such cases, polytherapy comes to practice where two or more drugs are simultaneously given to patients. The combination of local drug delivery of first line agents such as Phenobarbital and Phenytoin with newer antiepileptic drugs like Lamotrigine, Levetiracetam, Oxcarbazepine, etc., could revolutionize the treatment of refractory epilepsies (Aneja, Sharma, 2013). A study conducted to investigate the efficacy of combination therapy on 347 patients with refractory epilepsy revealed that when lamotrigine was added to valproate, carbamazepine and phenytoin monotherapy, the average seizure-free duration was 6.2 months on co-medication as compared to 2.1 months on monotherapy (Sarhan, Walker, Selai, 2015). Levetiracetam was also studied, and it proved to be a favorable agent with the best responder rate (efficacy measure) and withdrawal rate (García, 2016**)**. Literature reports a range of hyphenated techniques for estimation of antiepileptic drugs in plasma samples (Ferreira *et al.,* 2014*)* (Emami, Ghassami, Ahmadi, 2006). An analytical method for lamotrigine with 10 mM

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ammonium acetate (pH 3.0 ± 0.2 adjusted with formic acid): Methanol 70:30 (v/v) as the mobile phase with a flow rate of 1.0 ml/min is reported (Joshi *et al*., 2011). An analytical method for Levetiracetam in tablet and urine sample described by Tyagi *et al*., 2015, consisted of the mobile phase Methanol: Water: Triethanol amine 70:25:05 (v/v) with detection at 224 nm. An RP-HPLC method for detection of phenytoin in bulk and dosage form using a mixture of methanol and phosphate buffer (60:40 v/v) as the mobile phase at a flow rate of 0.7 ml/min and detected at 225nm was explained by Varaprasad *et al*. 2012. However, simultaneous determination of the drugs Levetiracetam (LVT), Lamotrigine (LTG), Phenobarbital (PBT) and Phenytoin (PTY) has not been previously reported in any literature. Therefore, it was thought of interest to develop and validate a suitable simple, precise and reproducible RP-HPLC method for simultaneous quantitation of selected antiepileptic agents in bulk as well as in formulation. Commercially, this will help reduce the cost of analysis by saving solvents for individual testing of drugs. The developed method can further be extrapolated to determine the test samples in blood plasma.

The drug Levetiracetam [(S)-2-(2-oxopyrrolidin-1-yl) butanamide] (PubChem CID 5284583) is a drug within the pyrrolidine class that has little-to-no potential to produce, or be subjected to pharmacokinetic interactions (Levetiracetam, cited 2017). It is indicated as an adjunctive therapy in the treatment of partial onset seizures in epileptic patients from one month of age. It is also used in the treatment of myoclonic seizures in patients with juvenile myoclonic epilepsy from 12 years and in primary generalized tonic-clonic seizures in patients with idiopathic generalized epilepsy. The drug is rapidly and nearly completely absorbed following oral administration, with a reported absolute oral bioavailability of essentially 100%. (Dooley, Plosker, 2000.). Lamotrigine [3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine] (PubChem CID 3878) is used for the treatment of epilepsy, bipolar disorder and pain syndromes and is effective against partial and secondarily generalized tonic-clonic seizures as monotherapy or adjunctive therapy(Leach, Marden, Miller, 1986). The drug is rapidly and completely absorbed from the gastrointestinal tract (Tmax, 1~3 h) with a bioavailability of approximately 98%, and steady-state serum concentrations increases linearly with dose. Phenobarbital [5-ethyl-5-phenyl-1,3-diazinane-2,4,6-trione] (PubChem CID 4763), the oldest modern antiepileptic drug discovered in 1912, was the first effective organic anti-epileptic agent against various forms of partial and generalized seizures but not against absence seizures. PHE is rapidly and completely absorbed from the gastrointestinal tract, with a bioavailability of 95~100% in adults, but in newborns, it exhibited delayed and incomplete absorption (Methaneethorn, Leelakanok, 2020). Phenytoin [5,5-diphenylimidazolidine-2,4-dione] (PubChem CID 1775) is believed to protect against seizures by causing a voltage-dependent block of voltage gated sodium channels. Oral bioavailability is 70-100%. Phenytoin binds preferentially to the inactive form of the sodium channel. Phenytoin sodium has some disadvantages, such as low treatment indexes, low security range and big differences among individuals. (Figure 1).

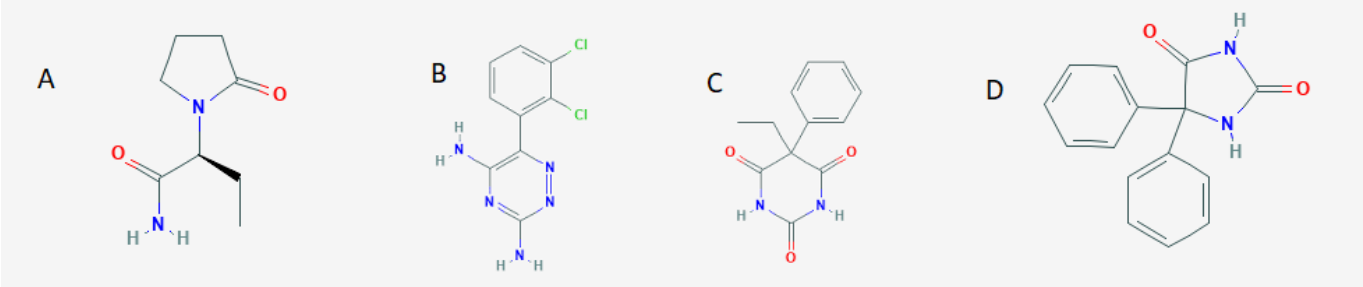

FIGURE 1 - A. Levetiracetam, B. Lamotrigine, C. Phenobarbital, D. Phenytoin.

The combination of local drug delivery of first line agents such as Phenobarbital, Phenytoin with newer antiepileptic drugs like Lamotrigine, Levetiracetam, Oxcarbazepine etc., could revolutionize the treatment of refractory epilepsies (Praveen, Panchaksharimath, Nagaraj, 2020) (Reddy, 2020). Literature survey reported quantitative estimation of the selected Antiepileptic agents Levetiracetam, Lamotrigine, Phenytoin and Phenobarbital individually in pharmaceutical formulation and in biological fluids using HPLC-MS/ UPLC (Ghatol *et al.,* 2013) (Hashem, El-Sayed, 2018); however, there were no methods available for simultaneous estimation of all four drugs together using simple analytical techniques like Reverse Phase HPLC UV. The aim of this work was to develop an RP-HPLC method for consistent simultaneous quantitation of four antiepileptic drugs Levetiracetam (LVT), Lamotrigine (LTG), Phenobarbital (PBT) and Phenytoin (PTY).

MATERIAL AND METHODS

Material

The test drugs Levetiracetam (assay ≥ 98%) from Hetero Labs Ltd, Lamotrigine (assay ≥ 98%) from CTX Lifescience Pvt. Ltd., Phenobarbital (assay ≥ 98%) from Harman Finochem Ltd. and Phenytoin (assay ≥ 98%) from Orgamine chemicals (I) Pvt. Ltd were kindly provided as gift samples. HPLC grade acetonitrile and methanol was purchased from Thomas Baker and Water HPLC grade from Anant Pharmaceuticals Pvt. Ltd. The branded tablets Levipil 250 (Levetiracetam 250mg), Lamepil 100 (Lamotrigine 100mg) and Garoin (Phenobarbital 50mg + Phenytoin 100mg) were procured from the local market.

Instrumentation and Chromatographic conditions

Chromatographic analysis was developed using Jasco HPLC with a UV detector equipped with Chromnav 2.0 software for recording and interpretation of data. The column used was Athena C_{18} (5µm C_{18}100 Å, LC Column 250 x 4.6 mm). Different combinations of mobile phase consisting of Water, Acetonitrile, 1mM Triethanolamine, 10mM Ammonium acetate buffer and 5mM KH_2PO_4 were tried (Rama *et al*., 2011; Kang *et al*., 2011) The mobile phase that gave the best resolution consisted of HPLC grade acetonitrile and phosphate buffer (pH 6 adjusted with 0.1N NaOH). The buffer was filtered through a 0.45µ Millipore filter and sonicated before use. The mobile phase i.e., acetonitrile and phosphate buffer, ACN: 5mM KH_2PO_4 (50:50) was delivered as an isocratic system at a flow rate of 1 ml/min and detection done at 230nm. The injection volume was 20µl.

Experimental Work

Preparation of standard stock

Accurately weighed 10mg of each drug Levetiracetam and Lamotrigine was dissolved in acetonitrile. Similarly, 10mg of each drug Phenobarbital and Phenytoin was dissolved in methanol to prepare 1000 ppm stock solution. For the preparation of a test solution of the marketed preparation, each marketed preparation was powdered, and tablet powder equivalent to 10mg of each drug was weighed and sonicated in the respective solvents, i.e., Levetiracetam and Lamotrigine in acetonitrile and Phenobarbital and Phenytoin in methanol to prepare 1000 ppm stock solution. The solutions were filtered and further diluted to prepare the stock solution of the marketed preparation.

Linearity curve of standard drugs

Calibration solutions were freshly prepared from 1000 ppm stock solutions of LVT, LTG, PBT and PTY. Further dilution from the stock solution was established using a mobile phase. Linearity was established in the following ranges LVT (20–70 ppm), LTG (1–6 ppm), PBT (5–30 ppm), PTY (10–60 ppm). A calibration curve of concentration v/s absorbance was plotted, and the linearity equation was determined.

Specificity

Specificity was studied to ensure that there is no interference in the developed analytical method, from

the excipients, that may be present in the marketed product. Specificity was evaluated by comparing the chromatograms of the standard solution of the drugs and the marketed product. An assay was performed on the marketed formulations, and drug content in the formulations was calculated from the linear regression data of the respective standards. For performing the specificity, from the stock solution (1000 ppm), a working solution (10 ml) of standard was prepared containing 30 ppm (LVT), 2 ppm (LTG), 10 ppm (PBT) and 20 ppm (PTY) in a mixture using the mobile phase as the diluent. Similarly, for the marketed preparation, the required amount was pipetted from the stock solution (1000 ppm) of the marketed sample and combined in a 10 ml volumetric flask, and the volume was made up to 10 ml mark with the mobile phase.

Method validation

The method validation was carried out according to the statistical method of validation of the ICH guideline Q2Q(R1) (Guidelines, ICH Harmonized Tripartite,1994). The method was validated for system suitability, precision, accuracy, robustness, limits of detection (LOD) and limits of quantification (LOQ). All validation studies were performed in triplicate. The system suitability was done by determining the peak area, retention time, theoretical factor and resolution for the four drugs. The ideal values for system suitability are resolution >1.5%, asymmetry factor <2 and theoretical plates >2000. The accuracy was determined by the percent recovery method and was performed on the marketed tablets. The sample solution contained tablet powder equivalent to 10 mg of the drug. i.e., 1000 ppm solution. The standard solution was spiked in this sample preparation of individual drugs at different levels i.e., 80%, 100%, 120%, thus making the overall concentration of the stock solutions at three different levels as 1800 ppm, 2000 ppm and 2200 ppm. The sample stock thus obtained was diluted further 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. System precision was done to ensure that the analytical system was working properly. For system precision, LVT 30 ppm, LTG 2 ppm, PBT 10 ppm and PTY 20 ppm was used; five injections of each sample were done and %RSD was calculated. Method precision was performed at three levels for intraday and inter day precision. The stock of each drug was diluted at 3 levels according to its linearity range i.e., LVT (30ppm, 50ppm, 70ppm), LTG (2ppm, 4ppm, 6ppm), PBT (10ppm,20ppm,30ppm) and PTY (20ppm, 40ppm, 60ppm), using the mobile phase as a diluent and was injected in triplicate on the same day for intraday precision and on different days for inter day precision, and %RSD was calculated from the results.

The sensitivity for the simultaneous determination of the four antiepileptic drugs was evaluated with respect to the LOD and limits of quantification LOQ. LOD and LOQ were calculated from the data derived from the calibration curve of each standard. These parameters demonstrate that the method used for analysis and quantification have adequate sensitivity to the concentration of the analyte. The slope average and standard deviation of the response were used to establish these parameters. According to the formula prescribed in the ICH guidelines, the LOD and LOQ were calculated for all the drugs. Robustness was evaluated by checking the effect of slight deliberate variation in the chromatographic parameters such as column temperature, mobile phase flow rate and mobile phase composition on retention time and peak area ratio. Change in pH was done by ±5%, and the assay was carried out at pH 5.95 and 6.05. Change in the flow rate was done by ±5% and the assay was carried out at 0.95ml/min and 1.05ml/min

RESULTS AND DISCUSSION

The drugs Levetiracetam and Lamotrigine are soluble in acetonitrile, while phenobarbital and phenytoin are soluble in methanol. In the developed RP-HPLC method, acetonitrile and phosphate buffer, ACN: 5 mM KH_2PO_4 (50:50) was delivered as an isocratic system at a flow rate of 1 mL/ min and detection done at 230nm. The reverse phase HPLC method described was validated as per ICH guidelines for determination of Levetiracetam, Lamotrigine, Phenobarbital, and

Phenytoin from bulk as well as from marketed formulation. The mean retention time for LVT, LTG, PBT and PTY were found to be 2.55, 3.55, 4.65 and 5.99 minutes respectively. The specificity of the method was evaluated by an assay of tablets (n=3), and it showed a percent content for Levetiracetam, Lamotrigine, Phenobarbital and Phenytoin as 90%, 90.3%, 93% and 103% w/w respectively, and they met the labeled claim of 90–110% as represented in Table I. The efficiency of the chromatographic separation was monitored by following the system suitability test parameters, namely resolution, tailing factor and the number of theoretical plates. The RP-HPLC method is considered suitable when the resolution is more than 1.5%, the tailing factor is less than 2 and theoretical plates more than 2000. All the drugs passed the acceptance criteria, and the results for them are represented in Table II. Further to it, there was no additional peak detected as an interference with the analyte peak as seen in the chromatogram, Figure 2. The range for the construction of the linearity curve for the four drugs could not be kept the same as the dose of the drug varied. However, the calibration curve was found to be linear in the stated range as mentioned in Table III. The LOD and LOQ were calculated as per the specified formula in the ICH guidelines and was found to be 2 ppm and 6.5 ppm for levetiracetam, 0.17 and 0.5 ppm for lamotrigine, 1.09 ppm and 3.3 ppm for phenobarbital and 3.02 ppm and 9.17 ppm for phenytoin respectively. System precision was checked by injecting the same concentration of the reference standard solution five times to ensure that the analytical system was working properly. The %RSD was calculated and was found to be 1.89% for Levetiracetam, 1.91% for Lamotrigine, 1.08% for Phenobarbital and 1.71% for phenytoin. The precision of the proposed method was checked by intra-day and inter-day repeatability of response by triplicate injections of the standard solution. The %RSD values were calculated and found to be <2.0 %; thus stating that the method is precise for the drugs in the given range as seen in Table IV. The accuracy of an analytical procedure is the closeness of the agreement between the value which is accepted as conventional true value or reference value and the value found. The accuracy of the proposed method was determined by the recovery studies. In the proposed method, accuracy was calculated by spiking the standard drug in the drug product at three different levels, i.e., 80%, 100%, and 120%. The accuracy was evaluated and was expressed as % recovery between the concentration established and the concentration added for all the drugs. All the accuracy values were within 85–115% of the recovery range as seen in Table V. Thus, our results suggested that the described method was accurate. For the evaluation of robustness, flow rate and pH were changed by ±5% and its effect on the retention time, tailing factor, NTP and peak area was observed. The method passed all the acceptance criteria and was found to be precise and robust as seen in Table VI and VII.

TABLE I - Specificity of the analytical method using marketed samples

Sr. No.	Peak Name	Retention time	Area	NTP	Resolution	Symmetry Factor	Assay
1	Levetiracetam	2.550	104614	2368	3.913	1.09	90.6%
2	Lamotrigine	3.558	134052	3489	4.150	1.45	90%
3	Phenobarbital	4.650	134819	4213	4.189	1.49	93%
4	Phenytoin	5.999	294096	4450	4.583	1.50	103%

TABLE II - System suitability parameters for the antiepileptic drugs

Parameter	Levetiracetam	Lamotrigine	Phenobarbital	Phenytoin	Acceptance limit
Rt	2.5min	3.5min	4.6min	5.9min	-
Symmetry factor	1.23	1.31	1.30	1.42	Not More Than 2
Resolution	5.171	4.502	4.608	4.812	Not Less Than 1.5

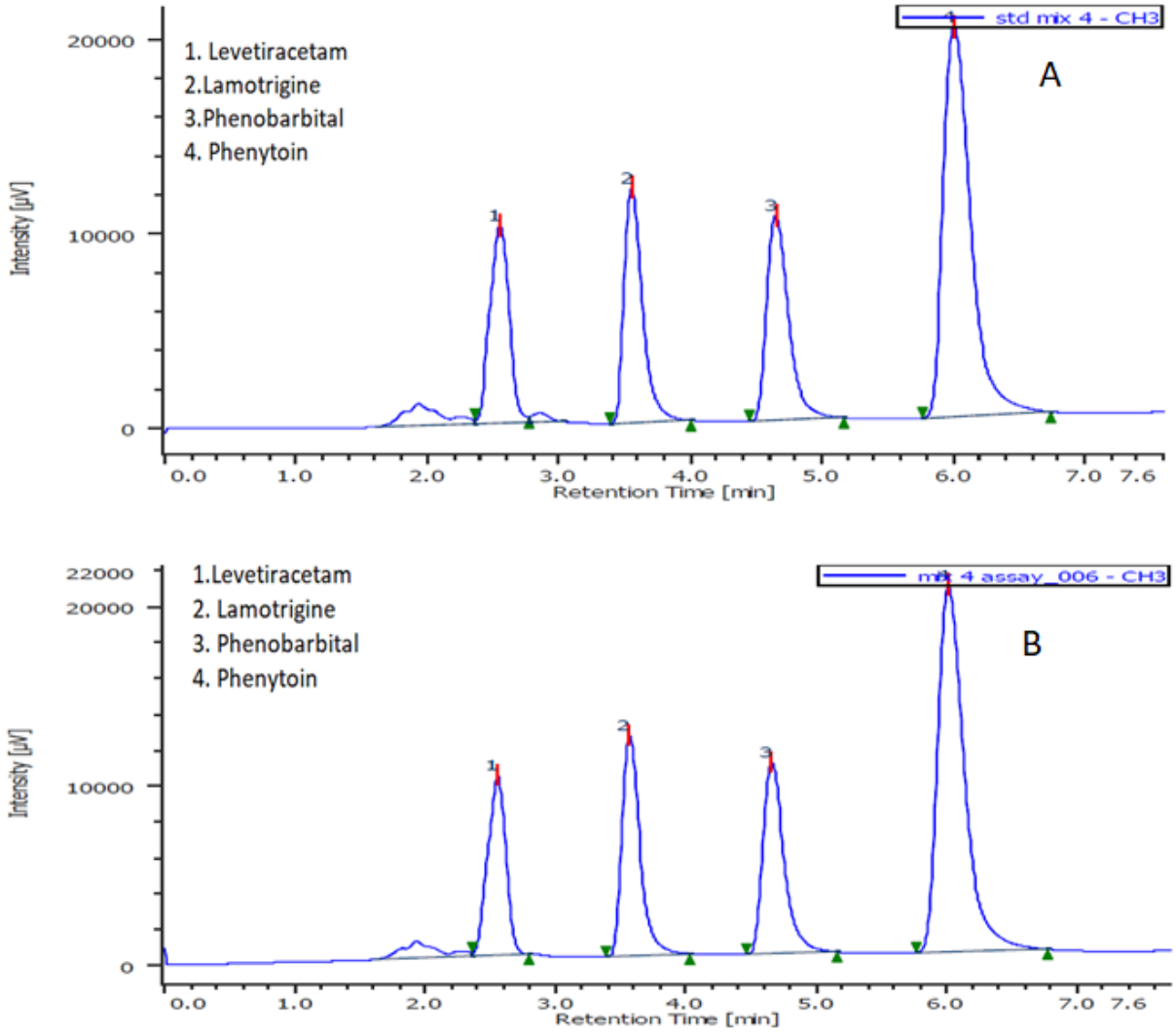

FIGURE 2 - HPLC Chromatogram of A standard drugs B. Marketed samples.

TABLE III - Linearity parameters for the antiepileptic drugs

Drug	Calibration range	Linearity equation	R^2
Levetiracetam	50-100ppm	y = 3812.x + 879.1	0.999
Lamotrigine	1-6ppm	y = 78023x - 6573.	0.999

TABLE III - Linearity parameters for the antiepileptic drugs

Drug	Calibration range	Linearity equation	R^2
Phenobarbital	5-30ppm	y = 14617x – 1770	0.998
Phenytoin	10-60ppm	y = 14472x – 4983	0.998

TABLE IV - Precision studies for the antiepileptic drugs

Parameter	Levetiracetam	Lamotrigine	Phenobarbital	Phenytoin
System Precision (%RSD)	1.89%	1.91%	1.08%	1.71%
Intraday precision Level 1	0.31%	1.5%	0.94%	1.3%
Level 2	1.13%	0.86%	1.21%	1.52%
Level 3	0.9%	1.37%	1.51%	0.77%
Inter day precision Level 1	1.98%	1.5%	1.51%	1.79%
Level 2	2.00%	1.73%	2%	1.88%
Level 3	1.62%	1.77%	1.95%	1.33%

TABLE V - Accuracy studies for the antiepileptic drugs

Drug	Levetiracetam (10mg)			Lamotrigine (10mg)			Phenobarbital (5mg)			Phenytoin (10mg)		
Spike level (%)	80	100	120	80	100	120	80	100	120	80	100	120
Amount present (mg)	18	20	22	18	20	22	9	10	11	18	20	22
Amount recovered (mg)	15.36	17.45	20.33	15.85	17.72	19.99	7.76	8.90	10.29	18.35	20.45	23.88
Percent recovery (%)	85.33	87.25	92.4	88.05	88.6	90.36	86.3	89.0	93.63	101.9	102.2	108.5
Average Percent recovery			88.32			89			89.64			104.2

TABLE VI - Robustness studies for antiepileptic drugs with change in flow rate

Drug	Levetiracetam (10mg)			Lamotrigine (10mg)			Phenobarbital (5mg)			Phenytoin (10mg)		
Flow rate (ml/min)	0.95	1	1.05	0.95	1	1.05	0.95	1	1.05	0.95	1	1.05
Retention time	2.6	2.5	2.4	3.6	3.5	3.3	4.8	4.6	4.3	6.1	5.9	5.6
Tailing factor	0.98	1.09	0.97	1.45	1.45	1.39	1.45	1.49	1.46	1.5	1.5	1.5
NTP	2100	2368.	2145	3908	3489	3721	4716	4213	4461	4981	4450	4787
Area	105194	104614	100535	129511	134052	119380	122644	134819	1216 09	285610	2940 96	2749 13
Assay **%**	91	90.6	87	85	90	80	85	93	84	100	103	96.5

TABLE VII - Robustness studies for antiepileptic drugs with change in pH

Drug	Levetiracetam (10mg)			Lamotrigine (10mg)			Phenobarbital (5mg)			Phenytoin (10mg)		
pH	5.95	6	6.05	5.95	6	6.05	5.95	6	6.05	5.95	6	6.05
Retention time	2.5	2.5	2.5	3.5	3.5	3.5	4.6	4.6	4.6	6.1	5.9	5.6
Tailing factor	0.98	1.09	0.92	1.45	1.45	1.45	1.42	1.49	1.45	1.5	1.5	1.5
NTP	2330	2368.	2111	3363	3489	3330	4403	4213	4350	4981	4450	4787
Area	99137	104614	103197	121307	134052	119923	125930	134819	1197 83	279361	294096	289156
Assay **%**	85.6	90.6	89.3	82	90	81	87	93	92	100	103	96.5

CONCLUSION

This research paper focuses on a novel method for simultaneous estimation and validation of Levetiracetam, Lamotrigine, Phenobarbital and Phenytoin using RP-HPLC-UV from bulk and formulations. Literature reports the simultaneous analysis of antiepileptic techniques using hyphenated techniques and using Photodiode-Array Detectors. (Sommerfeld-Klatta *et al.*, 2020). However, this research paper emphasizes on using simple RP-HPLC-UV for simultaneous analysis of two first generation and two second generation antiepileptics. All the experimental results were in the range of acceptable precision and accuracy, which indicate that the newly developed method is simple, specific, precise, accurate and robust. This method can be further extrapolated for analysis of these drugs in biological fluids and thus once validated can be used for Therapeutic drug monitoring.

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