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Stability indicating RP-HPLC method for estimation of levetiracetam in pharmaceutical formulation and application to pharmacokinetic study

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ABSTRACT

A stability-indicating HPLC method was developed and validated for the quantitative determination of Levetiracetam in tablet dosage forms. The separation was achieved using a Phenomenex® 250 mm × 4.6 mm i.d., 5 µm particle size C18 column. Mobile phase containing a mixture of Methanol, water and Acetonitrile (30:10:60 v/v) was pumped at a flow rate of 1.0 mL/min. UV detection was performed at 212 nm. The drug was subjected to acid, alkaline, oxidative, hydrolytic, photolytic and thermal degradation. Complete seperation of degraded product was achieved from parent compound. The chromatographic analysis time was approximately 7 min per sample with complete resolution of Levetiracetam (t_R = 5.351 min) from the major degradation products. The method was validated for accuracy, precision, specificity, linearity and sensitivity. The calibration plots were linear over the concentration ranges 0.01 – 1.5 µg/mL with limit of detection of 0.005 µg/mL and limit of quantification 0.01 µg/mL for Levetiracetam. The developed method was successfully applied for the precise analysis of the studied drug in its pharmaceutical dosage form. The mean percentage recovery was found to be 99.86 ± 0.4206 for levetiracetam. As the method could effectively separate the drug from its degradation products, it can be employed for analysis of stability samples. The developed method has been successfully applied for chemical kinetic study. The study shows zero order degradation behaviour.

Key Words: Levetiracetam; RP-HPLC; Pharmaceutical dosage form, stress degradation, chemical kinetics.

INTRODUCTION

The development of new antiepileptic drugs for epilepsy over the last decade has been spurred by the fact that the available antiepileptic drugs did not provide optimal care for patients with epilepsy[(1)]. Levetiracetam (LEVE) is a new antiepileptic drug that is currently used as an add-on therapy or monotherapy in patients with partial and secondary generalized seizures[(2)]. Levetiracetam (Figure 1), (-)-(S)-α-ethyl-2-oxo-1-pyrrolidine acetamide, $C_8H_{14}N_2O_2$, molecular weight 170.21 gm/mol is chemically unrelated with other antiepileptic drugs in current use, differing in structure and pharmacology.

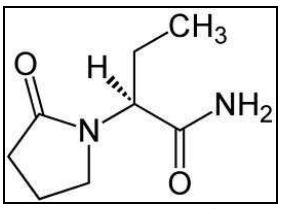

Figure 1: Chemical structure of Levetiracetam

Levetiracetam has a broad spectrum in antiepileptic activity[3, 4]. The efficacy of seizures treatment depends on the drug quality, which requires suitable monitoring. The quality controls of the anticonvulsant drugs are fundamental for the well-being of patients and are imperative for the development of routine analytical methods that can reliably measure these. Levetiracetam has been approved in the European Union as a monotherapy treatment for epilepsy in the case of partial seizures, or as an adjunctive therapy for partial, myoclonic and tonic-clonic seizures. It is also used in veterinary medicine for similar purposes. Levetiracetam has potential benefits for other psychiatric and neurologic conditions such as Tourette syndrome, autism, bipolar disorder and anxiety disorder. Along with other anticonvulsants like gabapentin, it is also sometimes used to treat neuropathic pain. *In vitro* and *in vivo* recordings of epileptiform activity from the hippocampus have shown that levetiracetam inhibits burst firing without affecting normal neuronal excitability, suggesting that levetiracetam may selectively prevent hypersynchronization of epileptiform burst firing and propagation of seizure activity.

Levetiracetam binds to synaptic vesicle protein SV2A, thought to be involved in the regulation of vesicle exocytosis. Although the molecular significance of levetiracetam binding to synaptic vesicle protein SV2A is not understood, levetiracetam and related analogs showed a rank order of affinity for SV2A which correlated with the potency of their antiseizure activity in audiogenic seizure-prone mice. These findings suggest that the interaction of levetiracetam with the SV2A protein may contribute to the antiepileptic mechanism of action of the drug. Levetiracetam is rapidly absorbed, with peak plasma concentrations occurring in about an hour following oral administration in fasted subjects. The oral bioavailability of levetiracetam tablets is 100% and the tablets and oral solution are bioequivalent in rate and extent of absorption. Food does not affect the extent of absorption of levetiracetam.

Assays reported in the literature for Levetiracetam determination in biological fluid and in tablet formulation includes high-performance liquid chromatography[8-13]. Shihabi ZK and collaborators[14] described a method for analysis of Levetiracetam tablets by capillary electrophoresis. Rao BM and collaborators[15] described a LC method for the enantioselective analysis of Levetiracetam and its enantiomer R-alpha-ethyl-2-oxo-pyrrolidine acetamide. A literature review revealed that an official monograph of Levetiracetam in tablets does not present in any Pharmacopoeia. Hence it was thought of interest to develop and validate a simple, economic and precise stability indicating HPLC method for the estimation of levetiracetam in pharmaceutical formulation and to extend the study by chemical kinetics.

MATERIALS AND METHODS

Materials and reagents

Levetiracetam standard were provided by Zydus Cadila Health Care, Ahmedabad, India. “Levexx - 250” tablet was procured from local market. Methanol was supplied by Merck Pvt. ltd, Mumbai. HPLC grade distilled water, Acetonitrile and all other reagents used in this study were of AR grade and were supplied by Merck Pvt. ltd, Mumbai.

Instrumentation and chromatographic conditions

The chromatographic separations were performed using Phenomenex® C18 (250 mm x 4.6 mm i.d, 5 µm particle size) column at 40 ºC temperatures. The optimum mobile phase consisted of methanol, water and acetonitrile in the ratio of 30:10:60. Auto sampler 20 µl was used and kept at 15 ºC temperature. Analysis was done with flow rate of 1.0 ml/min at 212 nm (λ max of Levetiracetam) wavelength by using photodiode array (PDA) detector.

Determination of wavelength of maximum absorbance

The standard solution of Levetiracetam was scanned over wavelength of 200 to 400 nm by using UV-Visible spectrophotometer and 212 nm was selected as analytical wavelength for analysis of levetiracetam in pharmaceutical dosage form as depicted in Figure: 2.

Preparation of mobile phase

HPLC grade solvents were used in separate bottle of gradient pumps as mobile phase. Methanol, water and acetonitrile in the ratio of 30:10:60 v/v were adjusted by gradient pump operated by LC solution software. Mixed solvents were degassed by the instrument and used as mobile phase.

Preparation of standard stock solution(100 µg/ml)

Accurately weighed 10 mg levetiracetam was transferred to 100 ml volumetric flask, dissolved in 50 ml methanol and diluted up to mark with methanol.

Preparation of standard working solution (10 µg/ml)

Accurately measured 10 ml of standard stock solution was transferred to 100 ml volumetric flask and diluted up to the mark with methanol to obtain 10 µg/ml of levetiracetam.

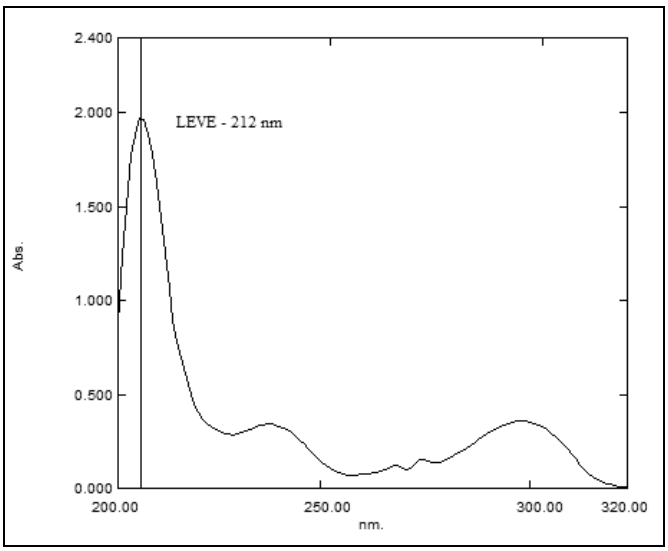

Figure 2: UV spectra of Levetiracetam between 200 and 400 nm by UV-Visible spectrophotometer.

Preparation of calibration curves of levetiracetam

Appropriate aliquots of standard working solutions of levetiracetam (0.01, 0.1, 0.2, 0.4, 0.6, 1.0 and 1.5 ml) were transferred to a series of 10 ml of volumetric flasks and diluted to the mark with mobile phase. The solutions were filled in the vials of auto sampler rack. Aliquots (20 µl) of each solution were injected from auto sampler under the operating chromatographic conditions described above. Calibration curve was constructed by plotting peak area versus concentration of levetiracetam and the regression equation was calculated as depicted in Figure 3.

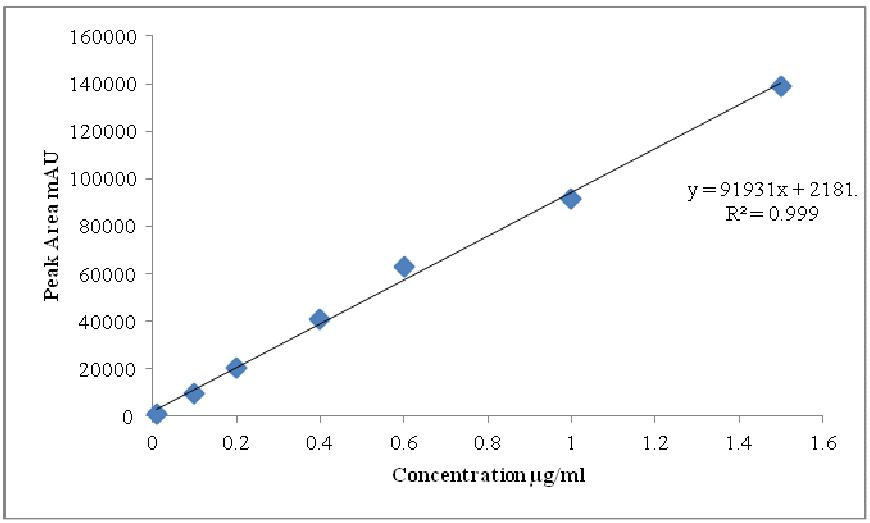

Figure 3: Calibration curve of Levetiracetam at 212 nm by RP-HPLC method

Preparation of sample solution (10 µg/ml)

Contents of twenty tablets "Levexx – 250"were accurately weighed and average weight per tablet was determined, contents were grounded to fine powder. An accurately weighed quantity of the pulverized powder equivalent to 10 mg of Levetiracetam was weighed and transferred to 100 ml volumetric flask, dissolved in methanol (60 ml) and sonicated for 30 min. The solution was filtered through Whatmann filter paper No. 41 and residue was washed with methanol. To ensure, the solution was filtered again through 0.45 µ filter paper. The solution was diluted up to the mark with methanol. Accurately measured 10 ml of standard sample stock solution was transferred to 100 ml volumetric flask, diluted up to the mark with methanol to get final working concentration of 10 µg/ml.

Analysis of Tablet dosage form
Appropriate three different aliquots from sample solution were suitably diluted with mobile phase in such a way to get concentrations in a range of 0.01 to 1.5 µg/ml for levetiracetam. The finally prepared solutions were analysed under chromatographic condition as described above. The amount of levetiracetam present in sample solution was determined by fitting the area response into the regression equation of levetiracetam in the method.

Force degradation study
In order to determine whether the analytical method and assay were stability-indicating, levetiracetam tablet powder was stressed under various conditions to conduct forced degradation studies. As levetiracetam is freely soluble in methanol, so methanol was used as solvent in all studies. All solutions for forced degradation studies were prepared to yield starting concentration 100 µg/ml of Levetiracetam. In all cases, tablet powder contents equivalent to 10 mg Levetiracetam was accurately weighed and prepared for analysis as previously described.

Acid degradation
Solution of levetiracetam (100 µg/ml) for acid degradation study was prepared using 0.1N hydrochloric acid in methanol and the resultant solution was refluxed at 60 ºC for 4 hours to facilitate acid degradation of levetiracetam.

Alkali degradation
Solution of levetiracetam (100 µg/ml) for alkali degradation study was prepared using 0.1N sodium hydroxide in methanol and the resultant solution was refluxed at 60 ºC for 4 hours to facilitate alkali degradation of levetiracetam.

Wet heat (Hydrolysis)
Solution of levetiracetam (100 µg/ml) for wet heat degradation study was prepared using triple distilled water and the resultant solution was refluxed at 60 ºC for 4 hours to facilitate hydrolysis of levetiracetam.

Oxidation
Solution of levetiracetam (100 µg/ml) for oxidation study was prepared using 3 % H_2O_2 in methanol and the resultant solution was refluxed at 60 ºC for 8 hours to facilitate oxidation of levetiracetam.

Dry heat
Solution for dry heat study were prepared by exposing approximately 50 mg of tablet powder in aluminium foil to dry heat in an oven at 60 ºC for 24 hour to facilitate dry heat degradation of levetiracetam. The tablet powder was removed from oven and the content of powder equivalent to 10 mg of levetiracetam was accurately weighed and transferred to volumetric flask and dissolved in methanol to make the final concentration 100 µg/ml of levetiracetam.

Photo stability[4] (Sun light and UV light)
Levetiracetam tablet powder was exposed to sunlight and UV radiation to determine the effects of light irradiation on the stability of Levetiracetam in the solid state. Approximately 100 mg of Levetiracetam tablet powder was spread on a glass dish in a layer that was less than 2 mm thick. All samples for photo stability testing were placed in direct sunlight exposed for 12 hours and under UV cabinet (At 25^0C with both UV radiation shorter and higher wavelength 210 and 310, respectively) for 12 hours. The tablet powder were removed from the sun and UV light and the contents of powder equivalent to 10 mg of Levetiracetam was accurately weighed and transferred to volumetric flask to make the final concentration 100 µg/ml of levetiracetam. Control samples which were protected with aluminium foil were also placed in the light cabinet and exposed concurrently. Following removal from the light cabinet, all samples were prepared for analysis as previously described.

Method validation
The developed analytical method was subjected to validation with respect to various parameters such as linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, recovery studies, specificity and reproducibility as per the ICH guidelines.

Linearity & range
The calibration curve was plotted over the concentration range of 0.01 to 1.5 µg/ml of levetiracetam. The calibration curves were prepared by plotting the peak area versus the concentration and analyzed through linear regression (Figure 4). The linearity was observed in the expected concentration range, demonstrating its suitability for analysis.

Accuracy (% Recovery)
The accuracy of the method was determined by calculating recoveries of levetiracetam by the standard addition method. Known amounts of standard solutions of levetiracetam (50, 100, and 150 % level) were added to previously

analyzed sample solutions of capsule dosage form. The amount of levetiracetam was analyzed by applying these values to the regression equation of the calibration curve. The experiment was conducted for three times.

Precision

Method precision (% Repeatability)

The precision of the instrument was checked by repeatedly injecting (n = 6) solution of levetiracetam (0.4 µg/ml). The results were reported in term of % coefficient of variance (% CV) should not more than 2 %.

Intermediate precision

Precision was evaluated in terms of intraday and interday precision. The intraday precision was investigated using three different concentrations of sample solutions prepared as discussed above, from stock solution. The intraday and interday precision of the proposed method was determined by analysing the corresponding concentration 3 times on the same day and on different days over a concentration of levetiracetam (0.6, 0.8, and 1.0 µg/ml). The results were reported in terms of % coefficient of variance (% CV).

Robustness

Method robustness was performed by applying small changes in the ratio of mobile phase, injection volume, and column temperature and flow rate. Robustness of the method was done at three different concentration levels of 0.6, 0.8, and 1.0 µg/ml. The results were expressed in terms of % CV

Limit of detection and quantification

The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected. and limit of quantitation (LOQ) of the method is the lowest concentration that can be measured with the precision and accuracy LOQ and LOD were calculated according to the following equation

$LOQ = 10\ \sigma/S$

$LOD = 3.3\ \sigma/S$

where σ is the standard deviation of intercept of regression line and S is the slope of the calibration curve.

Specificity and selectivity

The specificity of the method was established through resolution factor of the drug peak from the nearest resolving peak and also among all other peaks. Selectivity was confirmed through peak purity data using a PDA detector. To assess the method specificity, tablet powder without levetiracetam (placebo) was prepared with the excipients as for commercial preparation and compared with respective levetiracetam standard to evaluate specificity of the method. Representative chromatograms of placebo and standard were compared for retention time, resolution factor and purity.

System Suitability

The system suitability parameters like theoretical plates (TP), and asymmetry factor (As), capacity factor (K'), resolution (Rs), retention time (tR) and tailing factor (Tf) reported in European Pharmacopoeia were calculated by LC solution software.

Solution stability

The solution stability of levetiracetam in the assay method was carried out by leaving both the test and standard solution in tightly capped volumetric flask at room temperature for 24 hours. The same sample solutions were assayed for interval of 6 hours up to the 24 hours throughout the study period. Both the solution was prepared in methanol as solvent and mobile phase. The obtained results were compared with the freshly prepared solution.

RESULTS AND DISCUSSION

Method Optimization

The aim of this study was to develop a stability indicating HPLC assay method and its application in chemical kinetic study of levetiracetam. Initial studies to develop the method involved the use of C_{18} column with various mobile phases ratio containing methanol, water and acetonitrile. For the study concentration of 1.0 µg/ml for levetiracetam was prepared for all the standard and stressed samples. In almost every system studied, while the separation of more polar compounds was in some instance obtained, levetiracetam showed a retention time greater than 4 min. In this case, the optimized mobile phase was constituted by methanol:water:acetonitrile 30:10:60 v/v. Taken together these features provided chromatograms with good peak shape with a steady baseline required for the

analysis of levetiracetam in presence of degraded impurities with an acceptable retention time. It was observed that satisfactory resolution of levetiracetam and its degradation products formed under various conditions and present in the mixture of stressed samples were achieved. The method was optimized to separate major degradation products formed under various conditions. Resolution was also checked on mixture of the degradation samples to confirm the separation behaviour. It indicates that the developed method was successful in separation of drug and all chromophoric degradation products. The capacity factor *k* were 4.2, 5.1 and 6.5, for Impurity A, Impurity B and levetiracetam, respectively being the range within $2 < k < 10$. Generally to obtain the best chromatographic behaviour capacity factor of $2 < k < 10$ was desired. For quantitative determination, detection of levetiracetam and its impurities were performed using PDA detector. Each degraded impurities were detected at its wavelength maxima with 100 % purity. A representative chromatogram is shown in Figure 4, which satisfies all the system suitability criteria, better resolution of the peak from solvent peak with clear base line separation was found.

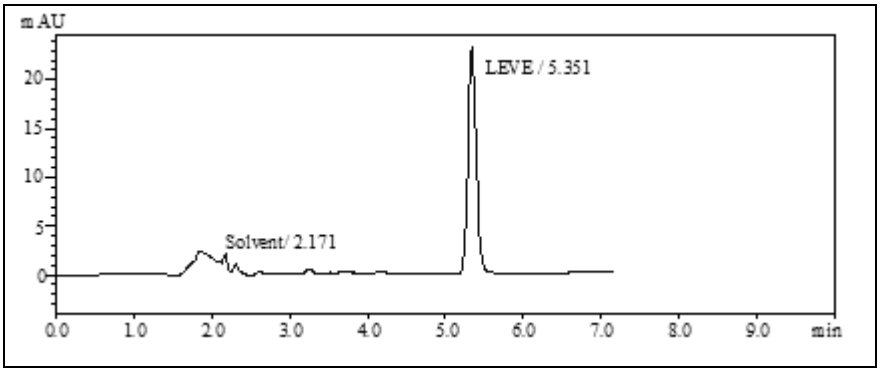

Figure 4: Chromatogram of Levetiracetam standard (1µg/ml) with corresponding retention time at 212 nm by RP-HPLC method

Force degradation of levetiracetam

Singh and Bakshi, on stress testing[6-7] suggested a target degradation of 20-80 % for the establishing stability indicating nature of the assay method, as even intermediate degradation products should not interfere with any stage of drug analysis. Though conditions used for forced degradation were attenuated to achieve degradation in the range of 20-80 %, this could not be achieved in some cases even after exposure for prolonged duration. Levetiracetam showed extensive degradation in acidic, basic, wet and oxidative condition within very short time. Table: 1. indicates the extent of degradation of levetiracetam under various stress conditions. *Stability data for the degradation of levetiracetam were analyzed according to the ICH guidelines*[8].

Degradation behaviour of levetiracetam

In total, three degradation products were detected by LC on decomposition of the drug under various stress conditions. Levetiracetam showed extensive degradation in acidic, basic, wet and oxidative condition. The degradation behaviour, retention time (RT) and relative retention time (RRT) of the drug and the degradation products are listed in Table: 1. The degradation behaviour of the drug in individual stress conditions is outlined below. HPLC studies of samples obtained on stress testing of levetiracetam under different conditions using methanol : water : acetonitrile (30:10:40) as the mobile solvent system suggested for the following degradation behaviour. Stability study in all the force degradation condition show formation of two common impurities at retention time of 3.188 and 3.885 for Impurity-A and Impurity-B, respectively.

Table: 1. Force degradation summary of Levetiracetam by stability indicating RP-HPLC method

Stress condition/ state	Time	% Assay of active substance	Retention time (RT)			Relative retention time (RRT)		
			IMP-A	IMP-B	LEVE	IMP-A	IMP-B	LEVE
Acidic 0.1 N HCL (60ºC)/solution	4 hr	66.565	3.188	3.881	5.343	0.657	0.745	1.0
Alkali 0.1 N NaOH (60ºC)/solution	4 hr	68.837	3.198	3.884	5.302	0.659	0.746	1.0
Wet heat (60ºC)/solution	4 hr	62.484	3.182	3.878	5.342	0.632	0.726	1.0
Oxidative 3% H_2O_2 (60ºC)/solution	8 hr	62.024	3.194	3.888	5.334	0.645	0.736	1.0
Dry hear (60ºC)/solid	24 hr	61.716	3.132	3.834	5.378	0.665	0.753	1.0
Photo light/solid	12 hr	66.343	3.185	3.879	5.320	0.643	0.734	1.0
UV Light 254 and 310 nm/solid	12 hr	69.956	3.184	3.864	5.302	0.641	0.732	1.0

Acid degradation

It was observed that around 30–40 % of the drug degraded on refluxing it in methanolic 0.1N HCl for 4 hr and there was corresponding formation of degradation products (Fig 5) as compared to the chromatogram of drug in formulation (Fig 4). Two new peaks were seen in the chromatograms of acid-degraded samples of levetiracetam, as

compared to the standard (Fig 4). This indicates that the drug degraded to low molecular weight compounds. The drug concentration gradually decreased with time on refluxing in 0.1N HCl and formation of degraded products increases as the time pass.

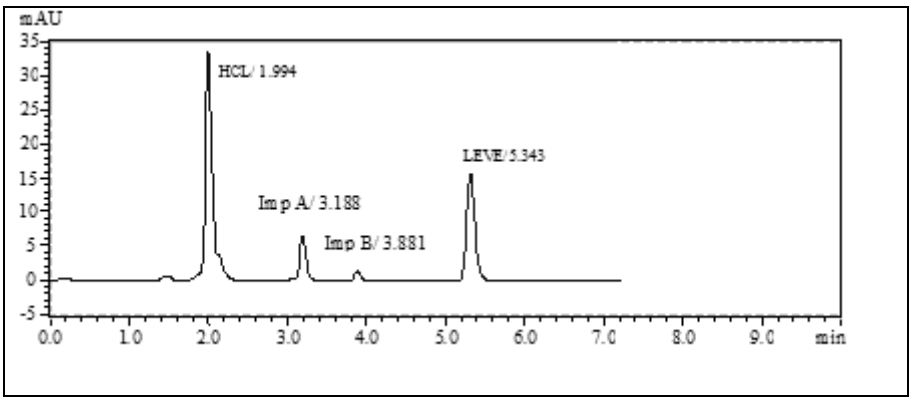

Figure: 5. Chromatogram of acid degradation of Levetiracetam at 212 nm by stability indicating RP-HPLC method

Alkali degradation

The results obtained on degradation of levetiracetam in alkaline conditions were found to be very similar to those reported for acidic condition. 30-40 % degradation was observed within 4 hr refluxing in methanolic 0.1N NaOH. Two new degraded impurities were separated from the drug. The product was seen during HPLC analyses of alkali-degraded samples with drug (Fig: 6) and compared with (Fig 4), chromatogram of Standard drug. The drug was found to be highly labile to alkaline condition. The rate of degradation in 0.1N NaOH was as fast as acid condition. Drug degradation was associated with rise in a major degradation product.

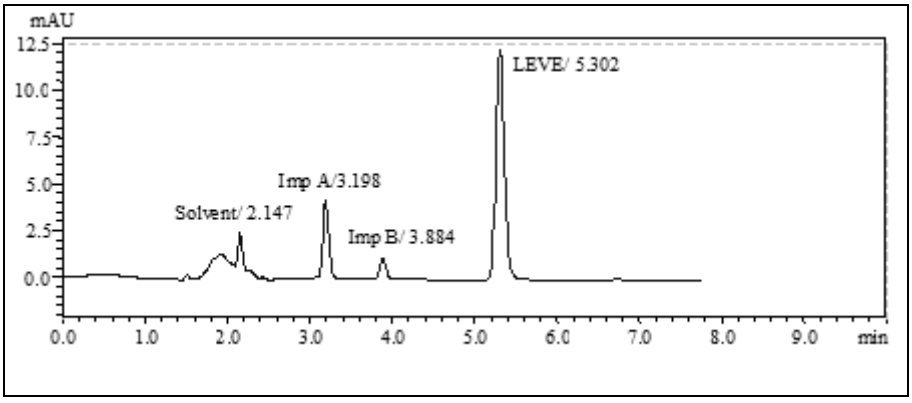

Figure: 6. Chromatogram of alkali degradation of Levetiracetam at 212 nm by stability indicating RP-HPLC method

Wet heat (Hydrolysis)

In neutral condition, only 20–25 % degradation of the drug was seen after refluxing at 60ºC for at the end of 4 h, degradation of the drug was observed with the corresponding rise in the major degradation peak (Fig 7). The degraded impurities observed in the wet degradation were not differing from the degradation of acid and alkali. The chromatogram was compared with standard and formulation.

Oxidation

Almost 25-35% drug degradation was observed on exposure to 3% H_2O_2 for 8 h. One major quantity degradation product peaks at around 3.194 minute was seen, and also there was significant rise in the height and area of these peaks with time (Fig: 8). The major peak observed at retention time of 2.001 RT was compared with blank chromatogram of 3% H_2O_2 indicates that peak was of H_2O_2 not of impurity. This showed that the drug was degraded in oxidative conditions, resulting in products resolving again at same RT as acid, alkali and wet degradation.

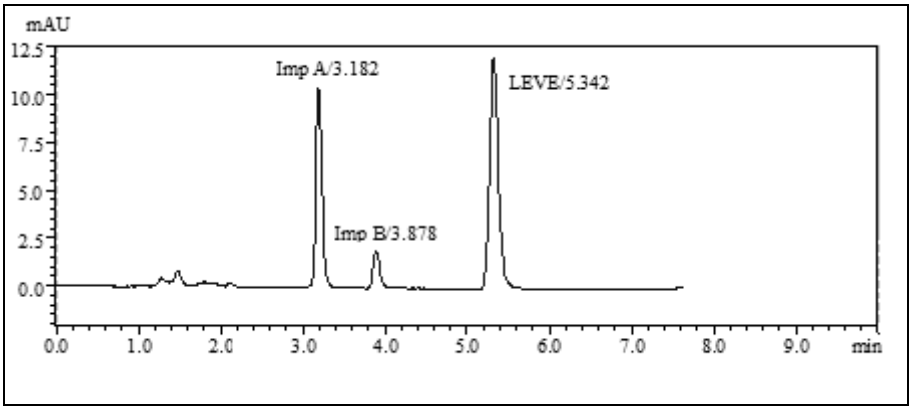

Figure: 7. Chromatogram of wet degradation of Levetiracetam at 212nm by stability indicating RP-HPLC method

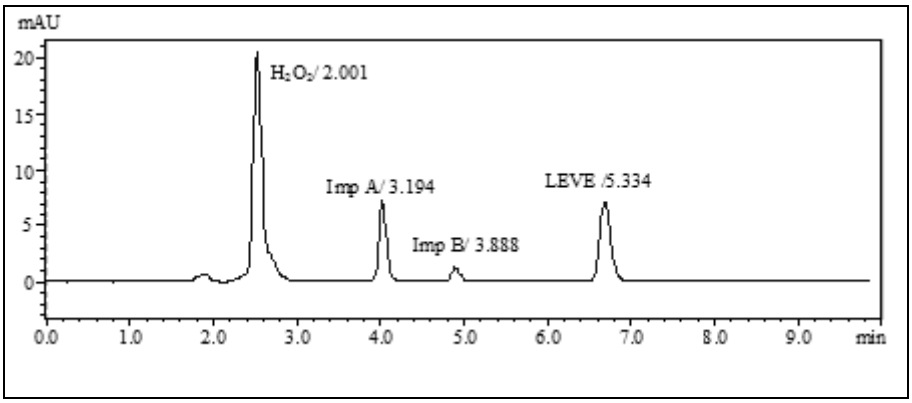

Figure: 8. Chromatogram of oxidative degradation of Levetiracetam at 212 nm by stability indicating RP-HPLC method

Dry heat

Levetiracetam was found to be stable as compare to acid, base and wet degradation after exposing the drug to 60ºC for 12 hours (Fig: 9). The exposure of the solid drug to 60ºC for 24 hours shows around 40 % degradation. It indicated that levetiracetam was stable to dry heat than other force degradation condition.

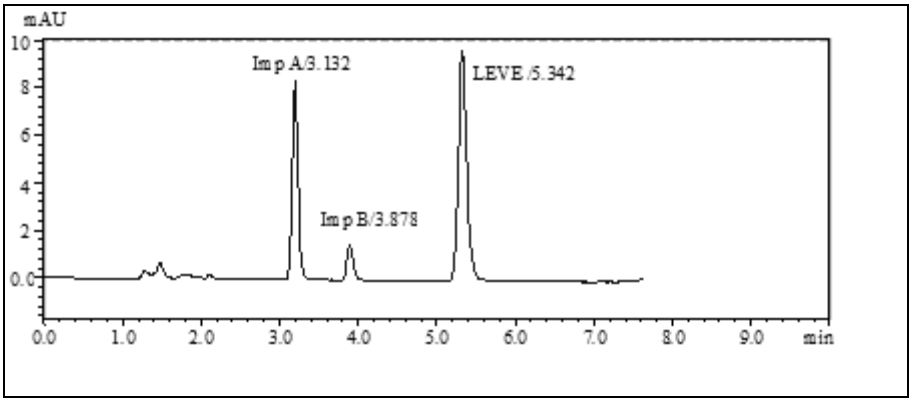

Figure: 9. Chromatogram of dry heat 60 ºC degradation of Levetiracetam at 212nm by stability indicating RP-HPLC method

Photo stability (Photo and UV light)

Levetiracetam was found to be labile on exposure to sunlight and UV light. In solid and methanolic condition 15-25 % degradation was observed. Under the conditions, the drug decomposed to the same major degradation product (Fig: 10 & 11), both were also formed during hydrolysis, alkali and acidic, and dry heat degradation. No degradation was observed in control samples in the dark chamber. Two degradation product was observed after exposure of drug solution in sunlight and UV light for 12 hours, only minor degradation products observed instead of major one as in other force degradation were formed. Almost 20-30% of the drug degraded in 12 hours with formation of a two minor degradation products between retention time ranges of 3 to 4 minute. This peak was found to increase with time. In this case, the fall in drug peak was in correspondence with the rise in degradation product peaks.

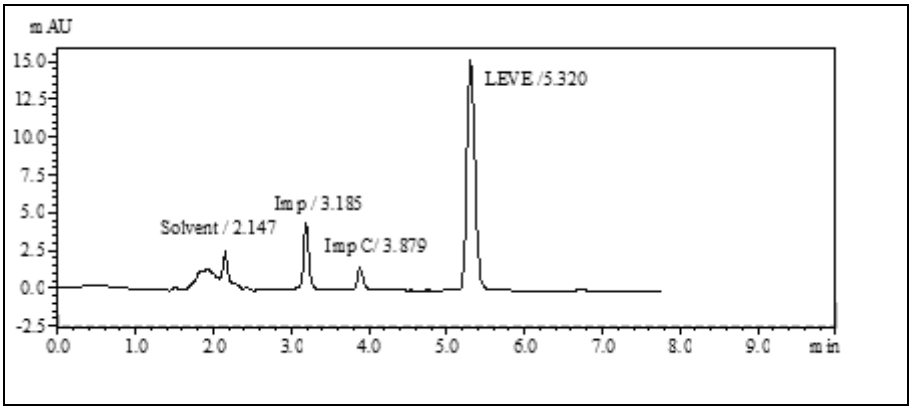

Figure: 10. Chromatogram of sunlight degradation of Levetiracetam at 212nm by stability indicating RP-HPLC method

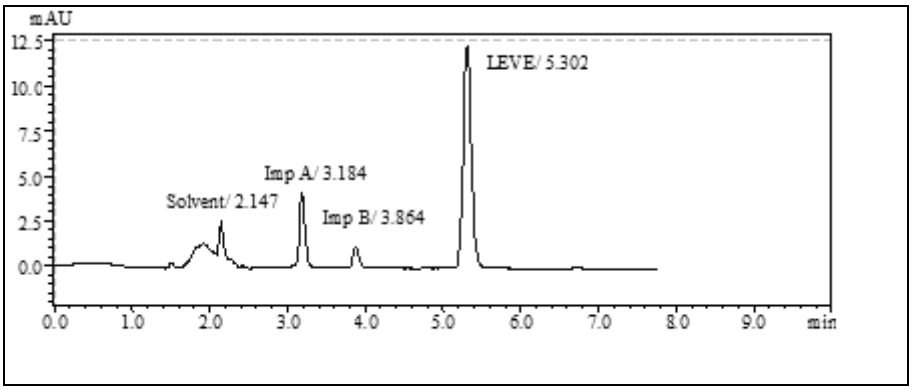

Figure: 11. Chromatogram of UV light degradation of Levetiracetam at 212nm by stability indicating RP-HPLC method

Linearity & range

Linearity of the method was evaluated at different concentration levels in the range from 0.01 – 1.5 µg/ml for levetiracetam. The results show that an excellent correlation existed between the peak area and concentration of analyte. The response of the drug was linear (r^2 = 0.999) for Levetiracetam in the concentration range between 0.01 – 1.5 µg/ml. Preparation of Solution for Calibration curve of Levetiracetam results obtained are shown in Table: 1 & 2.

Table 2: Result of calibration curve for Levetiracetam at 212 nm by stability indicating RP-HPLC method

Concentration (µg/ml)	Mean Peak Area ± Std. Deviation (n=6)	% CV
0.01	970.3333 ± 17.71	1.825793
0.1	9556.667 ± 114.06	1.193557
0.2	20396 ± 300.18	1.471775
0.4	40983.33 ± 521.63	1.272809
0.6	62909.5 ± 600.27	0.954184
1	91513.33 ± 884.98	0.967055
1.5	139200.8 ± 1145.22	0.822716

Table: 3. Statistical analysis of the calibration curve of Levetiracetam

Parameter	Levetiracetam
Linear Range (µg/ml)	0.01-1.5
Slope ± SD	91930.83 ± 606.5
Intercept ± SD	2181.3 ± 212.03
Correlation coefficient r^2	0.999
Limit of Detection (µg/ml)	0.005
Limit of Quantification (µg/ml)	0.01

Analysis of Tablet dosage form

The amount of levetiracetam present in sample solution was determined by fitting the area response into the regression equation of levetiracetam in the method. The results of the assay indicate that the method is suitable for the assay of Levetiracetam without interference from the excipients used in the tablet dosage form (Table: 4).

Table: 4 Assay of Levetiracetam in tablet powder by stability indicating RP-HPLC method

Levetiracetam µg/ml	Intra-day measured % Assay ± S.D[a], (n[b]=5)	Inter-day measured % Assay ± S.D[a], (n[b]=5)
0.5	101.266 ± 1.205	102.148 ± 1.123
1.0	102.133 ± 0.461	102.644 ± 0.431
1.5	100.444 ± 0.423	100.074 ± 0.332

Accuracy (% Recovery)

The accuracy of the method was determined by calculating recoveries of Levetiracetam by the standard addition method. Known amounts of standard solutions of Levetiracetam (50, 100, and 150 % level) added to previously analysed sample solution of tablet formulation followed by suitable dilution to obtain the required concentration range. The amount of levetiracetam were estimated by applying these values to the regression equation of the calibration curve. The resultant % CV for this study was found to be < 2.0 % with a corresponding percentage recovery value. The recoveries obtained for levetiracetam shown in Table: 5.

Table: 5. Accuracy (% Recovery) study of Levetiracetam by stability indicating RP-HPLC method

Formulation LEVEXX µg/ml	Added concentration µg/ml	Intra-day measured % recovery ± S.D[a], % CV[b] (n[c]=3)	Inter-day measured % recovery ± S.D[a], %CV[b] (n[c]=3)
0.4	0.2 (50%)	98.781 ± 0.322, 0.329	99.436 ± 0.301, 0.302
0.4	0. 4 (100%)	100.60 ± 0.469, 0.466	98.895 ± 0.487, 0.492
0.4	0.6 (150%)	100.20 ± 0.472, 0.471	101.43 ± 0.208, 0.205

S.D[a] = Standard deviation, % CV[b] = Coefficient of variance, n[c] = Number of replicate

Precision

Method precision (% Repeatability)

The precision of the method was checked by repeatedly injecting (n= 6) solution of Levetiracetam (0.4 µg/ml) and measuring respective retention time and peak area. The results were reported in term of coefficient of variance (CV) as depicted in Table: 6.

Table: 6. Method precision data of Levetiracetam by stability indicating RP-HPLC method

Levetiracetam 1.0 µg/ml (n[c]=6)	Retention time (Min.)	Area
1	5.312	91756
2	5.386	92057
3	5.351	91746
4	5.391	89967
5	5.369	92476
6	5.352	91078
Mean	5.360	91513.33
S.D[a]	0.028	884.984
% CV[b]	0.538	0.967

S.D[a] = Standard deviation, % CV[b] = Coefficient of variance, n[c] = Number of replicate

Intermediate precision

The intraday and interday precision of the proposed method was determined by analysing the corresponding concentration 3 times on the same day and on different days over a concentration of Levetiracetam (0.4, 0.6, and 1.0 µg/ml) and their results were reported in terms of coefficient of variance (CV) and depicted in Table: 7.

Table: 7. Intermediate precision data of Levetiracetam by stability indicating RP-HPLC method

LEVE µg/ml	Intra-day measured mean area ± S.D[a], %CV[b] (n[c]=3)	Inter-day measured mean area ± S.D[a], %CV[b] (n[c]=3)
0.6	61003.756 ± 867.786,1.422	60789.124 ± 968.583, 1.593
0.8	78576.782 ± 1023.657,1.302	77980.684 ± 1201.032, 1.540
1.0	91756.654 ± 1553.781,1.693	91,34.142 ± 1739.079, 1.910

S.D[a] = Standard deviation, % CV[b] = Coefficient of variance, n[c] = Number of replicate

Robustness

Method robustness was performed by applying small changes in the ratio of mobile phase, injection volume, and column temperature and flow rate. Robustness of the method was done at three different concentration levels of 0.6, 0.8, and 1.0 µg/ml. The results of change in ratio of mobile phase, column temperature, wavelength, and injection volume are shown in Table 8.

Table: 8. Intraday robustness data of Levetiracetam by stability indicating RP-HPLC method

Parameter	Modification	% Recovery ± S.D[a], % CV[b] (n[c]=6)
Flow rate (1 ml/min)	1 ± 0.1	100.654 ± 0.954, 0.947
Mobile phase composition Methanol:water:acetonitrile (30:10:60 v/v)	29:10:61	99.412 ± 1.231, 1.238
	31:10:59	99.422 ± 1.324, 1.331
Wave length (212 nm)	212 ± 1	100.124 ± 1.546, 1.544
Injection volume (20 µl)	20 ± 1	99.739 ± 1.741, 1.745
Column temperature (40° C)	40 ± 2	100.002 ± 1.855, 1.854

S.D[a] = Standard deviation, % CV[b] = Coefficient of variance, n[c] = Number of replicate

Limit of detection and quantification

The LOD and LOQ were found to be 0.005 and 0.01 µg/ml for the determination of levetiracetam respectively as shown in (Fig: 12 & 13). The lowest concentration of the range is selected as LOQ because it is the lowest concentration that can be measured with the precision and accuracy. These data show that the method is sensitive for the determination of levetiracetam.

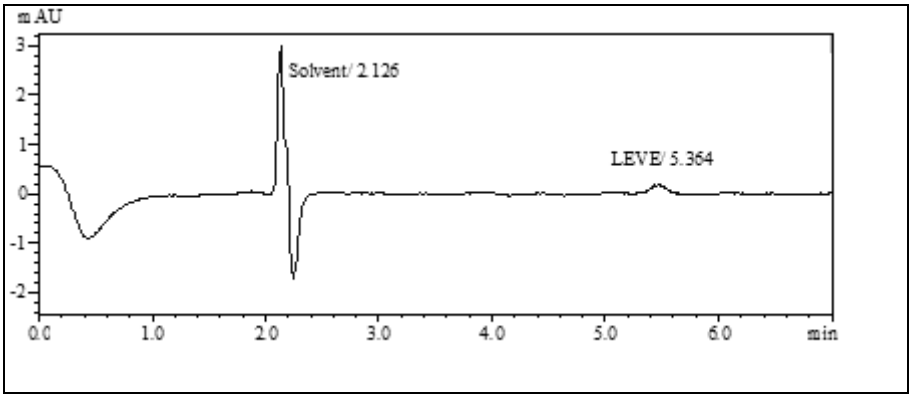

Figure: 12. Chromatogram for LOD of Levetiracetam at 212nm by stability indicating RP-HPLC method

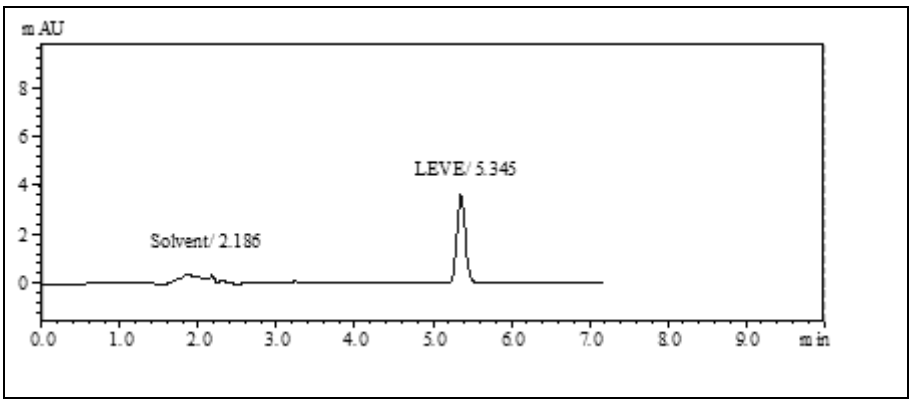

Figure: 13 Chromatogram for LOQ of Levetiracetam at 212nm by stability indicating RP-HPLC method

Table: 9. System suitability parameters of Levetiracetam by stability indicating RP-HPLC method

Parameter	Value		
	Levetiracetam	Impurity A	Impurity B
Retention time (Min.)	5.360	3.186	3.879
Resolution (Rs)	4.2	2.3	3.1
Theoretical plates (T_P)	5372	6677	6743
Tailing factor (T_f)	0.57	0.45	0.54
Asymmetric factor (A_f)	0.62	0.71	0.74
Capacity factor (K')	6.5	4.2	5.1

System suitability

The resolution factor for the levetiracetam from the nearest resolving solvent peak was > 3 in all samples. The placebo shows no detector response near retention time of 3.268 min, while the levetiracetam standard display good resoluted peak [Figure 3] and no interference from excipients present in the formulation [Figure 7] indicate specific

nature of the method. The purity curve and data of the levetiracetam shows that no other excipients are co-eluted with the drug and the peak is pure in nature.

Chemical kinetic study of Levetiracetam

In all the degradation condition, a decreasing in concentration was observed with increasing in time (Table: 10). Graphical representation of concentration versus time for all different condition (Fig: 14-17) shows that degradation follows zero order reaction, there is no effect of concentration on degradation was observed. Degradation of drug was independent of concentration of Levetiracetam. Also we can observe the degradation rate constant for zero order reaction (K_0) as slope of the plot. In acidic, alkaline and wet heat condition, degradation rate constant are same between 8 to 9. Oxidative degradation rate constant was found around 4. In remaining all condition rate constant were found to be between 1 to 2.

Table: 10. Chemical kinetic study of Levetiracetam by stability indicating RP-HPLC method

Time (hours)	Force degradation condition						
	1	2	3	4	5	6	7
	Concentration µg/ml						
0	100	100	100	100	100	100	100
1	91.586	92.574	-	-	-	79.462	-
4	66.384	67.048	82.475	88.65	86.384	60.576	94.653
8	27.76	32.473	63.572	76.07	86.384	21	89.633
10	8.047	18.573	-	-	-	3.573	-
12	-	-	42.684	67.003	69.467	-	76.56
18	-	-	-	46.31	44.534	-	66.473
20	-	-	3.795	-	-	-	-
30	-	-	-	12.463	13.735	-	54.603
48	-	-	-	-	-	-	35.657

1 = Acidic 0.1 N HCL (60ºC)/solution
2 = Alkali 0.1 N NaOH (60ºC)/solution
3 = Wet heat (60ºC)/solution
4 = Oxidative 3% H_2O_2 (60ºC)/solution
5 = Photo light /solid
6 = UV Light 254 and 310 nm/solid
7 = Dry hear (60ºC)/solid

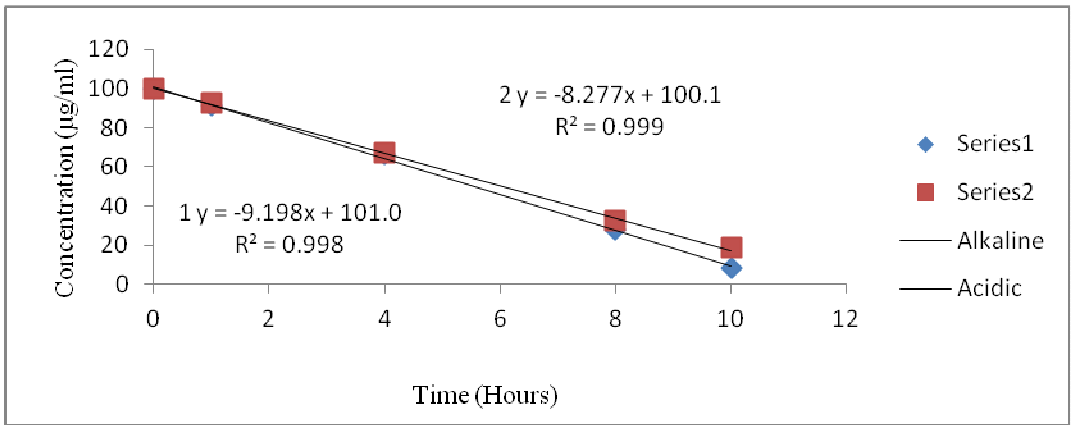

Figure: 14. Zero order plots for degradation of Levetiracetam in acidic and alkali condition

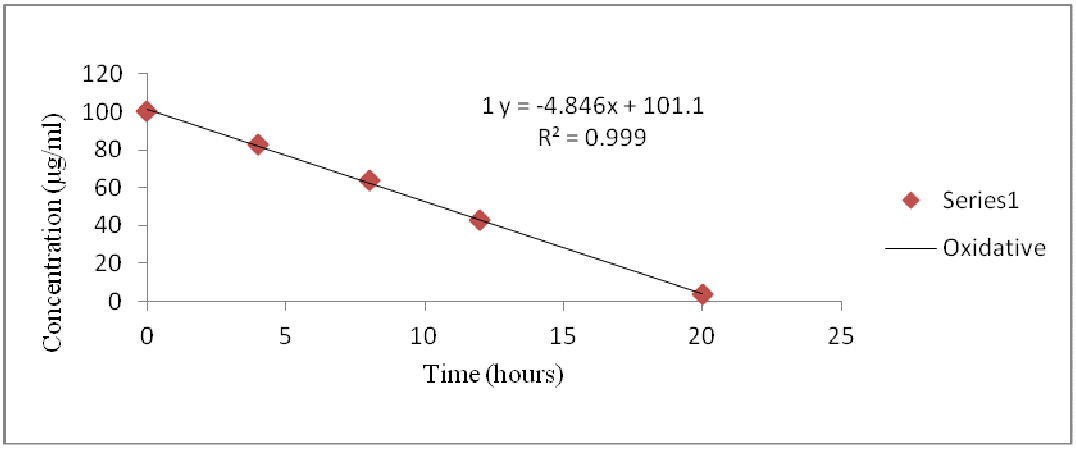

Figure: 15. Zero order plot for degradation of Levetiracetam in oxidative condition

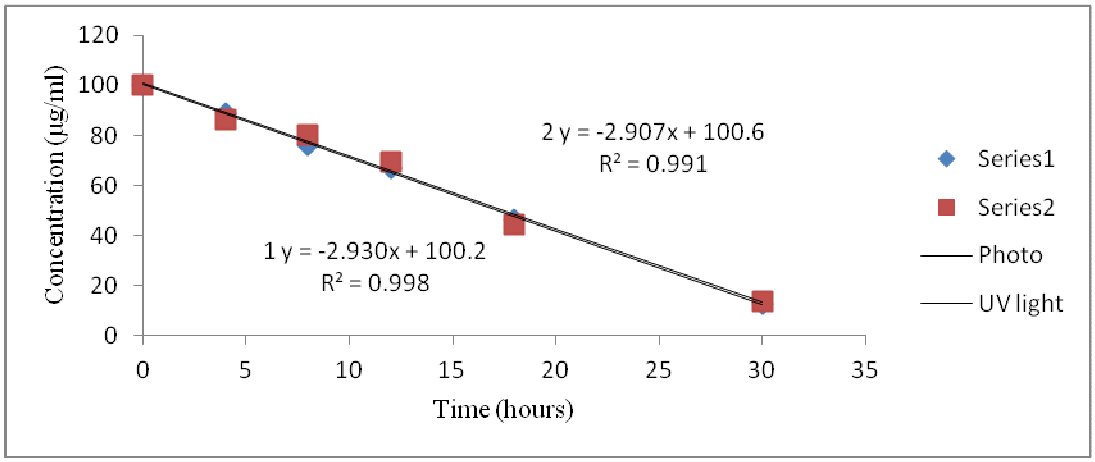

Figure: 16. Zero order plot for degradation of Levetiracetam in photo and UV condition

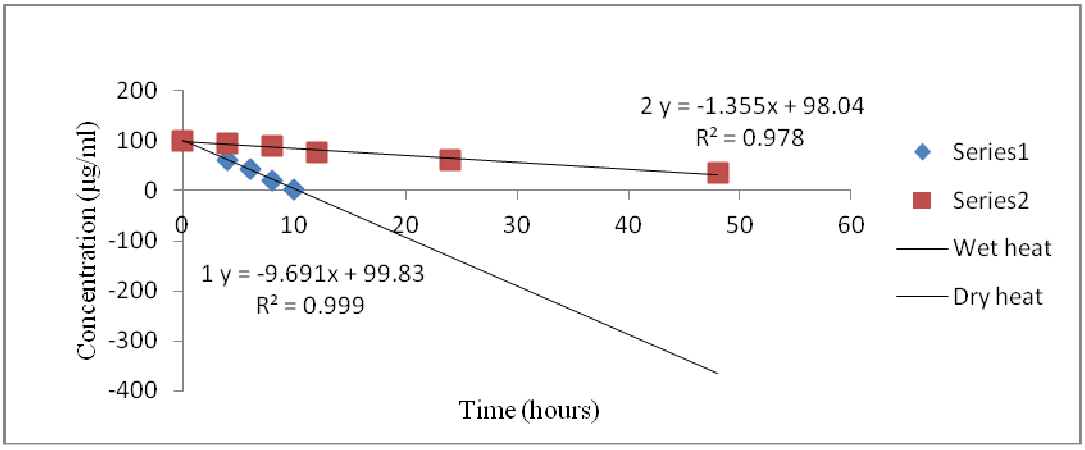

Figure: 17. Zero order plot for degradation of Levetiracetam in wet and dry heat condition

CONCLUSION

A validated stability-indicating RP-HPLC analytical method has been successfully applied for chemical kinetic study. The study shows zero oder degradation behaviour. The results of stress testing undertaken according to the International Conference on Harmonization (ICH) guidelines reveal that the method is selective and stability-indicating. The proposed method is simple, accurate, precise and has ability to separate drug from degradation products and excipients found in the dosage forms.

The RP-HPLC method developed meets the system suitability criteria, peak integrity and resolution from the parent drug and its degraded products. Two major degradation products were observed at RT 3.187, and 3.879 minute. Detection and quantification limits achieved, describe the method is very sensitive. High recovery and acceptable % CV values confirm established RP-HPLC method is accurate and precise. The complete separation of the analytes was accomplished within 7 minutes. The method has been successfully applied to perform accelerate stability study of Levetiracetam. Hence, the method is recommended for routine quality control analysis of Levetiracetam.

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