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Validated stability-indicating methods for determination of Levetiracetam

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ABSTRACT

New simple, accurate, rapid and reproducible methods have been developed and subsequently validated for the determination of Levetiracetam in presence of its acidic and alkaline-degradates, as stability-indicating studies. In the first method, two novel methods were adopted by utilizing zero-crossing technique, where the investigated drug was determined in presence of its both-degradates, by the use of third derivative (D^3) and second derivative ratio spectrophotometry (DR^2), respectively. The second method was isocratic reversed-phase (RP) stability-indicating high-performance liquid chromatographic (HPLC) method, which was adopted for determination of Levetiracetam in presence of its acidic and alkaline-degradates. The chromatographic separation was achieved isocratically using a mobile phase of acetonitrile: water (10:90, v/v), with 0.06 % triethylamine and pH adjusted to 2.5 using orthophosphoric acid. The analysis was carried out using Agilent eclipse XDB C18 column (150 mm × 4.6 mm, 5 µm) at flow rate of 1 $ml.min^{-1}$ and the UV detection at 205 nm. All the proposed methods were validated according to the International Conference on Harmonization (ICH) guidelines and successfully applied for determination of the Itopride Hydrochloride in pure form, in laboratory prepared mixtures and in pharmaceutical preparations. The obtained results were statistically compared to the reported method of analysis for Levetiracetam and no significant differences were found with respect to accuracy and precision.

KEYWORDS

Levetiracetam;
Derivative;
Derivative ratio spectrophotometry;
HPLC;
Stability indicating study.

INTRODUCTION

Levetiracetam, α-ethyl-2-oxo-1-pyrolidineacetamide[1-6], occurs as white to off white crystalline powder, with a faint odour and bitter taste; very soluble in water, freely soluble in chloroform and methanol, sparingly soluble in acetonitrile[7], having a molecular formula $C_8H_{14}N_2N_2O_2$ with molecular weight = 170.2[1]. It is an anti-epileptic drug and its mode of action is unknown[7-11].

The ICH-guidelines[12] requires performing stress-testing of the drug substance that can help in identifying the likely degradation-products, also can be useful in establishing the degradation-pathways and validating the

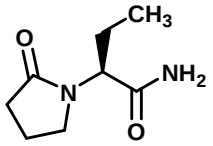

Figure 1 : Chemical structure of Levetiracetam

stability-indicating power of the analytical procedures used. Moreover, validated stability-indicating method should be applied in the stability study[13]. Stability-indicating methods can be specific one that evaluates the drug in the presence of its-degradation products, excipients and additives[14]. In this perspective, few analytical methods were employed for the determination of the investigated drug, including, high performance liquid chromatography[15-20].

These previous published methods comprise of complicated ones, therefore, the main aim of this work was to develop and validate stability indicating methods for estimation of Levetiracetam in the presence of its acidic and alkaline-degradates, which can be used for the routine quality control analysis of these drugs in raw material and pharmaceutical dosage form.

MATERIALS AND METHODS

Chemicals and reagents

Levetiracetam was kindly supplied by APIIC Industrial Estate, and certified to contain 99.95 %. Teratam® tablets: batch number: 82488 manufactured by Al-Andalous medical company and obtained from the local market. Each tablet was labeled to contain 500 mg of Levetiracetam.

Acetonitrile, methanol and bi-distilled water (Riedel-dehaen, Sigma-Aldrich, Germany), Hydrochloric acid (Adwic), aqueous 5.0M, sodium hydroxide (Adwic) aqueous, 5.0M, chloroform (Adwic), O-phosphoric acid (Adwic), triethylamine (Fluka) and TLC aluminium plates pre-coated with silica gel 60 F_{254} (E.Merck).

All chemical and reagents used through this work are of spectroscopic and chromatographic analytical grade. Bi-distilled water is used throughout the whole work and is indicated by the word 'water'.

Instruments

A double-beam Shimadzu (Japan) UV-VIS Spectrophotometer (UV-1601 PC), model TCC-240 A; connected to an IBM compatible computer and HP 695 C DeskJet printer is used. The bundled software is UVPC personal spectroscopy software version 3.7 (Shimadzu). The spectral bandwidth is 2 nm and the wavelength scanning speed was 2800.0 $nmmin^{-1}$. The absorption spectra of the reference and the test solutions are recorded in1.0-ml quartz cells at 25.0°C, using '$\Delta\lambda$ = 8 nm and scaling factor of 100 for second (D^2) and third (D^3) derivatives'.

The HPLC (Agilent Hewlett Packard series) instrument was equipped with a model series 1100 pump, manual injector Agilent 1100 series, 20 µl loops and a UV-visible wavelength detector Agilent 1100 series. The chromatographic separation was performed using (150×4.6 mm I.D.) Agilent eclipse XDB C18 and 5µm particle size at ambient temperature. Ultrasonic vibrator, (J.P Selecta'S-a; CD 300513 Espain). Disposible membrane filters, 0.45µm, (Agilent 3150-0576).

A pH-meter (Jenway 3510, UK), equipped with combined glass electrode for pH adjustment.

Standard solutions

(A) Standard solutions of Levetiracetam

Stock standard solutions of Levetiracetam, having concentration of (50.0 and 10 $\mu g.ml^{-1}$) were prepared in 0.05M HCl for spectrophotometric and chromatographic methods, respectively, which is used also as working standard solutions.

(B) Standard solution of degradates

1 **Standard solution of acidic-degradates:** Stock standard solution of Levetiracetam acidic-degradate was prepared by refluxing (0.0125 gm and 0.001gm) of Levetiracetam with 10.0 ml of 5.0 N HCl for 1.5 hours at 100°C, cooling, neutralizing the media with 5.0M NaOH and making volume to (250 ml and 100) with 0.05M HCl for spectrophotometric and chromatographic methods, respectively, which is used also as working standard solutions.

2 **Standard solution of alkaline-degradates:** As discussed under 2.3.2.1., but with using 5.0M NaOH for induction of degradation for 3 hours and 5.0M HCl for neutralization.

Complete degradation was checked by using TLC system; silica gel 60 F254 plates and chloroform: methanol (90: 30, v/v) as a developing system.

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TABLE 1a : Validation report of the proposed third derivative (D^3) spectrophotometric methods for the determination of Levetiracetam

Parameters	method D^3 in the presence of acidic-deg	method D^3 in the presence of alkaline-deg
Linearity	4.0-26.0μgml^{-1}	4.0-26.0μgml^{-1}
Slope	0.0269	0.0269
Intercept	0.0277	0.0277
Correlation coefficient (r)	0.9993	0.9993
Accuracy[a]	99.99 ±1.156	99.99±1.156
Specificity[b]	100.56±1.413	98.84±0.558
Precision		
Repeatability[c] 'intra-day'	0.909	0.909
Intermediate precision[c] 'inter-day'	0.973	0.973

[a]Mean ± RSD (n = 6), [b]Mean ± R.S.D.% (n = 6), [c]Mean ± R.S.D.% (n = 9)

TABLE 1b : Validation report of the proposed second derivative ratio spectrophotometric methods (DR^2) for the determination of Levetiracetam

Parameters	method DR^2 at 228.00nm	method DR^2 at 235.70nm
Linearity	4.0-22.0μg.ml^{-1}	2.0-22.0μg.ml^{-1}
Slope	0.104	0.026
Intercept	0.093	-0.0146
Correlation coefficient (r)	0.9994	0.9996
Accuracy[a]	100.24±1.181	99.76±0.656
Specificity[b]	99.43±1.272	98.87±1.310
Precision		
Repeatability[c] 'intra-day'	1.320	0.334
Intermediate precision[c] 'inter-day'	1.575	0.406

[a]Mean ± RSD (n = 6), [b]Mean ± RSD% (n = 6), [c]Mean ± RSD% (n = 9)

TABLE 1c : Validation report of the proposed UV-HPLC method for the determination of Levetiracetam

Parameters	Method In presence of Acidic-degradates	Alkaline-degradates
Linearity	0.5-3.5 μg.ml^{-1}	
Intercept	63.866	
Slope	-1.2229	
Correlation coefficient (r)	0.9995	
Accuracy[a]	100.14±1.161	
Precision		
Repeatability[a]	0.962	
Intermediate precision[a]	0.846	

[a]Mean ± R.S.D.%

Procedures

(A) Spectrophotometric determination of Levetiracetam in presence of its acidic and alkaline-degradates

(a) Third (D^3) derivative spectrophotometric method

From standard working solution of Levetiracetam, aliquots were transferred into a series of 25 ml volumetric flasks, and diluted to volume with 0.05M HCl to obtain a concentration range of 4-26 μg.ml^{-1}. The values of the third (D^3) derivative spectrophotometry amplitudes were computed for the investigated drug in presence of its acidic and alkaline-degradates, at 217.60 nm. Those values were then plotted versus corresponding concentrations; and the regression equation was then computed.

(b) Second (DR^2) derivative ratio spectrophotometric method

Calibration curve was performed by transferring aliquots of Levetiracetam stock standard solution into a series of 25 ml volumetric flasks, and diluting to volume with 0.05M HCl to obtain a concentration range of 4-22 μg.ml^{-1} and 2-22 μg.ml^{-1}, respectively. The spectra of acidic and alkaline-degradated solutions having concentration 6.0 μg.ml^{-1} were scanned and stored in the instrument PC as a devisor. The spectra of Levetiracetam were divided separately by the devisor's spectra and then the second derivative of the ratio spectra (DR^2) were computed for the investigated drug in presence of its acidic and alkaline-degradates, at 228.00 nm and 235.700 nm, respectively. Those values were then plotted versus corresponding concentrations; and the regression equation was then computed.

(B) Chromatographic determination of Levetiracetam in presence of its acidic and alkaline-degradates

Stationary phase, XDB C18 column (5μm, 150 × 4.6 mm), acetonitrile: water 'pH 2.5' in a ratio (10:90, v/v) with a flow rate was 1.0 ml.min^{-1} as 'degassed and filtered' mobile phase and UV detection at 205 nm, were the chromatographic conditions adopted. Construction the calibration curves were performed by transferring aliquots of Levetiracetam stock standard

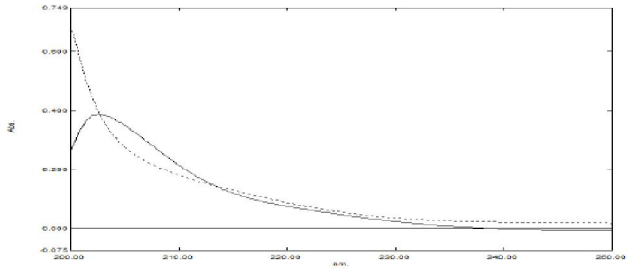

Figure 2a : Zero order absorption spectra of Levetiracetam (-) and its acidic-degradates (…), [each, 12.00µg.ml^{-1}]

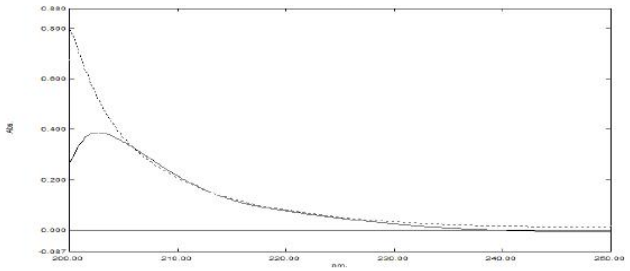

Figure 2b : Zero order absorption spectra of Levetiracetam (-) and its alkaline-degradates (…), [each, 12.00µg.ml^{-1}]

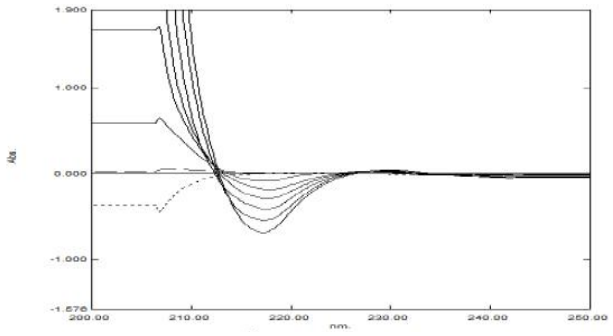

Figure 2c : Third derivative spectra (D^3) of Levetiracetam (-), its acidic (…) and alkaline-degradates (- - -)

TABLE 2 : Statistical comparison between the proposed [D^3, DR^2, UV- HPLC and] methods and the manufacturer's method* for determination of Levetiracetam

Parameters	Methods				
	D^3	DR^2 at 228.00nm	DR^2 at 235.70nm	U.V-HPLC	Reported Method*
Mean	98.47	98.75	98.14	98.69	98.804
S.D.	0.247	0.517	0.099	0.154	0.507
N	10	10	10	5	5
Variance	0.061	0.268	0.995×10^{-2}	0.023	0.257
t-test	-1.541	-0.179	-2.873	-0.497	-
Cal. t-value	2.571	2.306	2.306	2.31	-
F-test	0.238	1.041	0.039	0.093	-
Cal. F-value	0.275	6.388	0.157	0.156	-

Values in parenthesis are the theoretical values of *t* and *F* at P = 0.05. *The reported method is the HPLC method; lichrofspher column, acetonitrile: phosphate buffer pH 5.6 (15:85, v/v), as a mobile phase with a flow rate 0.8 ml.mint^{-1} and detection at 220 nm

solution into a series of 10 ml volumetric flasks and diluting with the mobile phase to the volume, having a concentration range of 0.5-3.5 µg.ml^{-1}. Under the previously mentioned chromatographic conditions, 20.0-µl volume from each solution was injected in triplicate, the average peak area obtained for each concentration was plotted versus concentration and the regression equation was then computed.

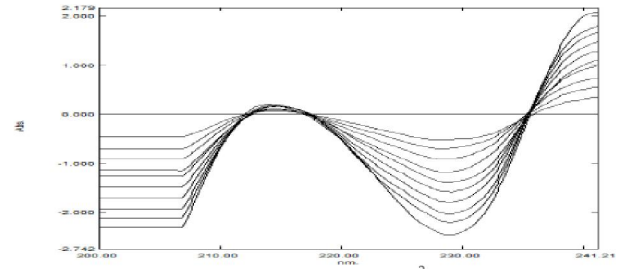

Figure 3a : Second derivative of derivative ratio spectra (DR^2) for different concentrations (4.0- 22.0 µg.ml^{-1}) of Levetiracetam at 228.00 nm, using 6.0 µg.ml^{-1} of its acidic-degradate as a divisor

(C) Assay of the pharmaceutical preparations

Twenty tablets of Teratam® were individually weighed to get the average weight of the tablets and finely powdered, respectively. Samples of the powdered tablets, claimed to contain 12.50mg and 1.0mg of Levetiracetam were transferred to 250 ml and 100-ml volumetric flasks for spectrophotometric and chromatographic methods, respectively, sonicated for one hour with 25 ml of acetonitrile: water 'pH 2.5' solvent, in a ratio (10:90, v/v), then the volume was brought to 250 ml and 100ml with 0.05M HCl, filtered to prepare stock standard solution and then the procedures mentioned under (A) and (B) were adopted. The concentrations of Levetiracetam were calculated from the regression equation.

RESULTS AND DISCUSSION

Method development

(A) Spectrophotometric method

(a) Derivative spectrophotometric method

The UV-spectra of Levetiracetam and its acidic and alkaline-degradates showed overlapping as shown in

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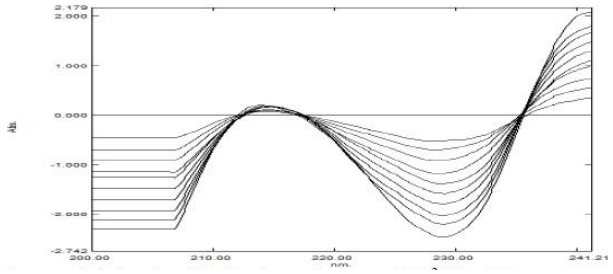

Figure 3b : second derivative of derivative ratio spectra (DR^2) for different concentrations (2.0- 22.0 µg.ml^{-1}) of Levetiracetam at 235.70 nm, using 6.0 µg.ml^{-1} of its alkaline as a divisor

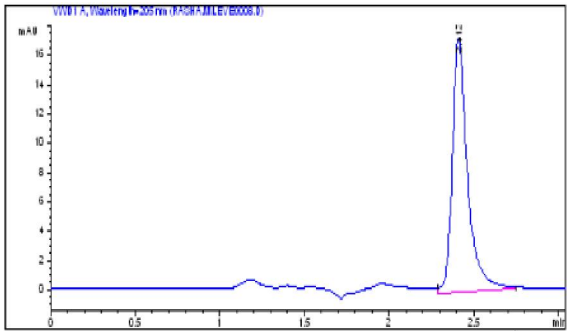

Figure 4a : HPLC chromatogram of Levetiracetam solution 2.00 µg.ml^{-1}

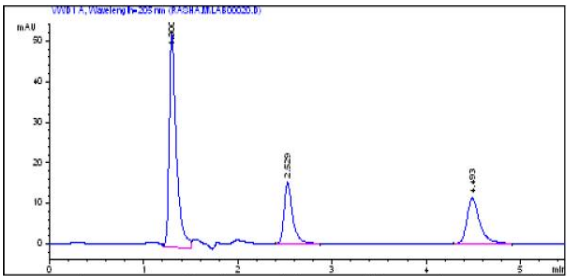

Figure 4b : HPLC chromatogram of a mixture solution containing Levetiracetam with its acidic-degradates (each, 2.50 µg.ml^{-1})

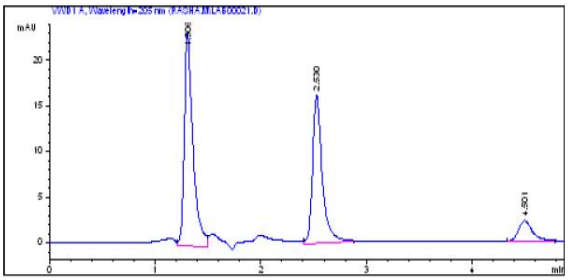

Figure 4c : HPLC chromatogram of mixture solution containing Levetiracetam with its alkaline-degradates (each, 4.00 µg.ml^{-1})

TABLE 3a : Results of the laboratory prepared mixtures for Levetiracetam with its acidic and alkaline-degradates by the proposed spectrophotometric method

Sample no.	Levetiracetam µg.ml^{-1}	Acidic &alkaline degradate µg.ml^{-1}	% Recovery *			
			In the presence of its acidic-degradates		In the presence of its alkaline-degradates	
			D^3 247.20 nm	DR^2 228.00 nm	D^3 298.20 nm	DR^2 235.70 nm
1	14.00	6.00	99.79	101.67	99.76	99.78
2	14.00	8.00	101.07	99.94	98.42	100.03
3	14.00	10.00	98.17	100.11	98.69	98.44
4	14.00	12.00	101.89	100.44	98.92	98.99
5	14.00	14.00	101.89	99.05	98.43	98.71
	Mean±R.S.D.%		100.56±0.1.413	100.24±0.944	98.84±0.558	99.19±693

*Mean of three determinations

figure 2a and 2b, which would not permit zero-order determination of Levetiracetam in the presence of its degradates, so derivative spectrophotometric methods were adopted, where zero-crossing point for acidic and alkaline-degradate of Levetiracetam were indicated. The third (D^3) derivative spectrophotometric technique permitted a selective determination of Levetiracetam in the presence of its acidic and alkaline-degradates at exactly the same wavelength 217.60 nm, as shown in figure 2c. The corresponding regression equations were found to be:

$$D^3_{217.60} = 0.0269C + 0.0277 \quad r = 0.9993$$

where, $D^1_{217.6}$ is the peak amplitudes at 217.60nm, C are the concentration of Levetiracetam in µg.ml^{-1} and r is the correlation coefficient.

(b) Derivative of ratio spectrophotometric method (DR^n)

The main advantage of derivative ratio spectra method (DR^n) might be the chance of taking measurement in correspondence to peaks and that the whole spectrum of interfering substance is cancelled, thus the wavelength

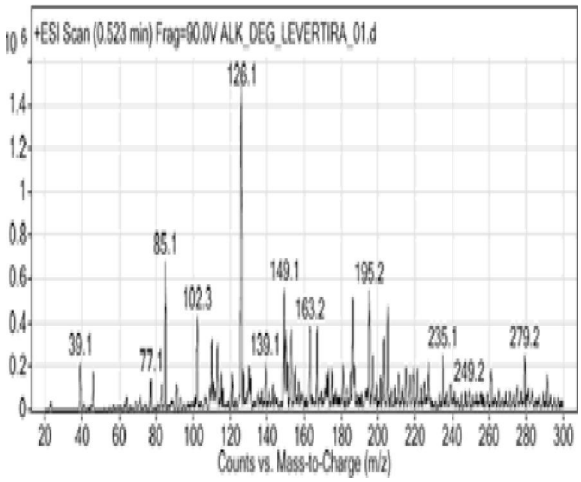

Figure 5 : LC-MS of acidic-degradates of Levetiracetam

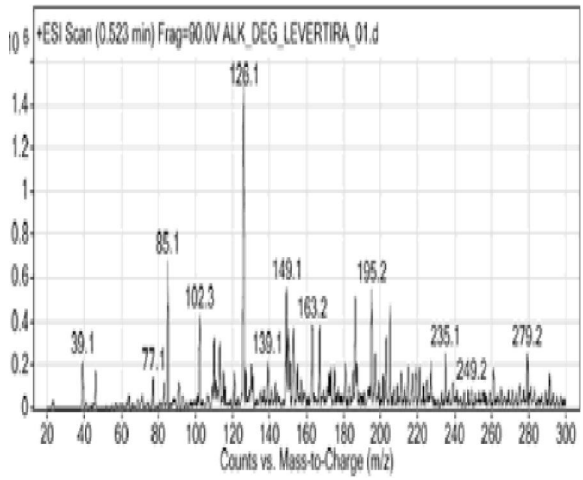

Figure 6 : LC-MS of alkaline-degradates of Levetiracetam

TABLE 3b : Results of the laboratory prepared mixtures for Levetiracetam with its alkaline-degradates by the proposed chromatographic method

Sample no.	Levetiracetam µg.ml^{-1}	Acid / alkaline-degradates µg.ml^{-1}	% Recovery *	
			U.V-HPLC (acidic)	U.V-HPLC (alkaline)
1	2.5	0.50	100.18	100.56
2	2.5	1.00	98.00	100.21
3	2.5	1.50	100.32	98.33
4	2.5	2.00	100.54	98.81
5	2.5	3.00	99.04	98.90
	Mean±R.S.D.%		99.60±1.06	99.76±0.617

*Mean of three determinations

TABLE 4a : Results from robustness testing of the proposed spectrophotometric and chromatographic methods for determination of Levetiracetam

Methods		Robustness (mean ±RSD)
Spectrophotometric methods	D^3	98.89±0.656
	DR^2 in presence of acidic-deg	100.28±1.819
	DR^2 in presence of alkaline-deg	99.38±0.267
UV-HPLC	UV detection	100.55±0.287

TABLE 4b : Results of system suitability of the proposed chromatographic method for determination of Levetiracetam

Parameters	Levetiracetam	limit
Retention time (t_R)	2.52	-
Resolution (Rs)	8.94	Rs >2
Tailing factor (T)	1.25	T < 2
Capacity factor (K')	3.98	1< K' <10
Selectivity factor (α)	1.92	α > 1
Column efficiency (N)	4936	N > 2000

selection for calibration is not critical. The main instrumental parameter conditions were optimized for a reliable determination of the compounds. Different divisor concentrations of acidic and alkaline-degradates were examined to select an appropriate concentration, which is very important factor in practice, where the best results were obtained by using 6.0 µg.ml^{-1} of acidic and alkaline-degradates, respectively as divisors. The second derivative ratio spectra (DR^2) at 228.00 and 235.70 nm permitted a selective determination of Levetiracetam in the presence of its acidic and alkaline-degradates as shown in (Figure 3a and 3b), where no noises were observed from the selected divisors. The corresponding regression equations were found to be:

$$DR^2_{228.00} = 0.1042C - 0.0928 \quad r = 0.9995$$

$$DR^2_{235.70} = 0.0261C - 0.0147 \quad r = 0.9993$$

where, $DR^2_{228.00}$ and $DR^2_{235.70}$ are the peak amplitudes at 228.00nm and 235.70nm, respectively, C is the concentration of Levetiracetam in µg.ml^{-1} and r is the correlation coefficient.

(B) Chromatographic method

The separation of Levetiracetam from its degradation-products has been performed on XDB C18 column. The proportion of the mobile phase components was optimized to reduce each of 'retention time and tailing' and to enable good resolution from its-degradates. Several trials were carried out to obtain good and optimum separation of Levetiracetam from its degradation products. Different composition mobile phases with different ratios were tried such as methanol: water (70 : 30, v/v), and acetonitrile: water (20:80, v/v), but the best resolution was obtained upon using acetonitrile: water

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TABLE 5a : Determination of Levetiracetam in pharmaceutical preparations[a] by the proposed spectrophotometric methods and application of standard addition technique, in presence of its acidic and alkaline-degradates

Pharmaceutical preparations	Claimed	% Found ± R.S.D*			Standard addition technique			
						%Recovery[b]		
		D^3	DR^2 at 228.00	DR^2 at 235.70	Added ($\mu g.ml^{-1}$)	D^3	DR^2 at 228.00	DR^2 at 235.70
Teratam® tablets B.N: 82488 [a]	500 mg	98.47 ± 0.252	98.75± 0.524	98.14± 0.101	4.00	101.05	98.57	101.32
					5.00	101.90	98.33	98.23
					6.00	101.14	98.73	100.08
Mean ± RSD%						101.36±0.461	98.88±0.407	98.88±1.573

[a]Teratam® tablets (Batch no: 82488) (labeled to contain 500 mg Levetiracetam per tablet). [b]Mean of three determinations

pH 2.5 (10:90, v/v) adjusted by using O-phosphoric acid with a flow rate of 1.0 $mlmin^{-1}$ and a detection wavelength 205 nm, where the maximum sensitivity was observed. The average retention time was 2.20 ± 0.05 min as shown in figure 4a-4c. The regression equation was computed and found to be:

A = 63.866C-1.2229 r = 0.9995

where, A is the relative peak area; C is Levetiracetam concentration in $\mu g.ml^{-1}$ and r is the correlation coefficient.

(C) Method validation

ICH guidelines[12] for validation method were followed, where all validation parameters were shown in TABLE 1a-1c. All the obtained results were statistically compared to the reported[7] method of Levetiracetam and no significant differences were found (TABLE 2).

(D) Specificity

Degradation behavior of Levetiracetam was investigated by the proposed spectrophotometric and chromatographic methods, where the investigated drug was determined in solutions containing different amounts of its acidic and alkaline-degradates by spectrophotometric method using (D^3), and (DR^2) techniques and by chromatographic method using [UV and fluorescence detection] techniques, respectively. Its Recovery % and R.S.D. % proved the high specificity of the adopted methods, where Levetiracetam could be determined in the presence of its acidic and alkaline-degradates (up to 100 %), as shown in TABLE 3a -3b.

(E) Robustness and system suitability of the HPLC method

The robustness of an analytical procedure is a measure of its capacity to remain unaffected after slit but deliberate changes in the analytical conditions. Separation of studied drug from its different degradates was performed under these conditions. In the propped spectrophotometric methods, the parameters of robustness were done in alterations of the used solvent and wavelengths, while in chromatographic methods; the alterations were done in wavelengths, flow rate, composition in mobile phase, and PH, (TABLE 4a). The system suitability parameters of HPLC method were evaluated[21] (TABLE 4b).

(F) Standard addition technique

To check the validity of the proposed methods, the standard addition method was applied by adding each drug to the previously analyzed tablets. The recovery of it was calculated by comparing the concentration obtained from the spiked samples with that of the pure drug. The results of analysis of the commercial tablets and the standard addition method (recovery study) of Levetiracetam are shown in TABLE 5a-5b suggested that there is no interference from any excipients, which are normally present in tablets.

(G) Identification of acidic and alkaline-degradates of Levetiracetam by structure elucidation

In this work, we were concerned with the acidic and alkaline-degradation of Levetiracetam, as it is completely degraded in a short time, which is considered a new way for determination of Levetiracetam in presence of its acidic and alkaline- degradates by using spectrophotometric methods.

Levetiracetam was influenced by refluxing with 5.0M HCl for 1.5 hrs at 100°C, giving two acidic-degradates, also, it was subjected to alkaline stress-testing by refluxing with 5M NaOH for 3 hours at 100°C, giving the same exactly degradation products as acidic hydrolysis. The identity of the acidic and alka-

TABLE 5b : Determination of Levetiracetam in pharmaceutical preparations[a] by the proposed chromatographic methods and application of standard addition technique

Pharmaceutical preparations	Claimed	% Found ± R.S.D*		Standard addition technique
		U.V-HPLC	Added ($\mu g.ml^{-1}$)	%Recovery[b] U.V-HPLC
Teratam® tablets B.N: 82488 [a]	500 mg	98.69±0.156	0.5	98.63
			1.00	99.02
			2.00	99.11
Mean ± RSD%				98.92±0.257

[a]Teratam® tablets (Batch no: 82488) (labeled to contain 500 mg Levetiracetam per tablet). [b]Mean of three determinations

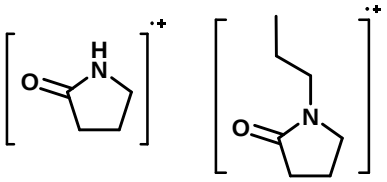

Figure 7 : Levetiracetam degradation products

line-degradates was confirmed by adopting LC-MS for each one, where the molecular ion peak of Levetiracetam 170.2 m/z was completely disappeared and two new molecular ion peaks were delivered when it was subjected to acidic and basic hydrolysis and hydrolysis of Levetiracetam corresponding to 85and 126 m/z, were appeared as shown in figure 5 and 6.

CONCLUSION

The proposed methods are accurate, precise and specific ones, where Levetiracetam can be determined in bulk powder and in pharmaceutical preparations without interference from common excipients present, also it can be determined in the presence of its acidic and alkaline-degradates. ICH guidelines were followed throughout the study for method validation and stress testing, and the suggested methods can be applied for routine quality control analysis and stability studies.

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