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A validated chiral LC method for the enantioselective analysis of Levetiracetam and its enantiomer *R*-α-ethyl-2-oxo-pyrrolidine acetamide on amylose-based stationary phase

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Abstract

A new, simple chiral HPLC method was developed for the enantiomeric separation of Levetiracetam, [(*S*)-α-ethyl-2-oxo-pyrrolidine acetamide], an antiepileptic drug in pharmaceutical formulations and in bulk materials. Enantiomeric separation was achieved on a chiralpak AD-H column using a mobile phase consisting of hexane and isopropanol in the ratio (90:10, v/v) at a flow rate of 1.0 ml/min. The resolution between the enantiomers was found to be not less than 7 in the optimized method. Interestingly, unwanted enantiomer, namely *R*-α-ethyl-2-oxo-pyrrolidine acetamide ((*R*)-enantiomer), was eluted prior to its mirror image in the developed method. The developed method was found to be selective in the presence of related impurities of Levetiracetam, namely *N*-(1-carbamoyl-propyl)-4-chloro-butyramide (Imp-1) and 1-ethyl-2-oxo-1-pyrrolidine acetic acid (Imp-2), and also under exposed conditions of UV light and 60 °C. The limit of detection (LOD) and limit of quantification (LOQ) of (*R*)-enantiomer were found to be 900 and 2250 ng/ml, respectively, for 10 μl injection volume. The method precision for (*R*)-enantiomer at limit of quantification level was within 8% R.S.D. Calibration curve for (*R*)-enantiomer was linear over the studied ranges (2250–9000 ng) with correlation coefficient greater than 0998. The active pharmaceutical ingredient was extracted from its finished dosage form (tablet) using isopropanol. The percentage recoveries of (*R*)-enantiomer were ranged from 94.2 to 102.6 and from 93.5 to 104.1 in spiked bulk and formulation samples of Levetiracetam, respectively. Levetiracetam sample solution and mobile phase are found to be stable for at least 48 h. The developed method was found to be rugged and robust. The proposed method was found to be suitable and accurate for the quantitative determination of (*R*)-enantiomer in bulk drugs and commercial formulations. Chiralcel OD-H column can also be used as an alternative column for the above purpose.
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Keywords: Levetiracetam; *R*-α-Ethyl-2-oxo-pyrrolidine acetamide; Enantiomer separation; Chiral HPLC; Validation; Chiralpak AD-H; Chiralcel OD-H

1. Introduction

Levetiracetam ($C_{18}H_{14}N_2O_2$), a single enantiomer (−)-(*S*)-α-ethyl-2-oxo-1-pyrrolidine acetamide, is a new antiepileptic drug for the adjunctive therapy

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of chronic epilepsy in adults [1,2]. Epilepsy is the most common neurological disorder of the brain. It is characterized by recurrent seizures due to excessive electrical activity in the brain. For patients who have insufficient seizure control with their current antiepileptic drugs, Levetiracetam is a highly effective antiepileptic agent with a wide margin of safety and straightforward pharmacokinetics that distinguish it from other currently available antiepileptic drugs [3,4]. FDA has approved Levetiracetam in May 2000 for marketing the drug worldwide [5].

A gas chromatographic-mass spectrometric method was reported in the literature for the enantioselective analysis of Levetiracetam and its enantiomer *R*-α-ethyl-2-oxo-pyrrolidine acetamide ((*R*)-enantiomer) in dog plasma and urine in biological samples [6]. This method involves a solid-phase extraction procedure followed by gas chromatographic separation of enantiomers on a chiral cyclodextrin capillary column and detection using ion trap mass spectrometry.

The general analytical techniques employed for the chiral separations include gas chromatography, high performance liquid chromatography (HPLC), and capillary electrophoresis.

Among the above-said techniques, HPLC is a versatile tool for the separation and quantification of enantiomers. So far to our present knowledge, no chiral HPLC methods were reported in the literature for the enantiomeric separation of Levetiracetam in bulk drugs and in pharmaceutical formulations.

Separation of enantiomers has become very important in analytical chemistry, especially in the pharmaceutical and biological fields, because some stereoisomers of racemic drugs have quite different pharmacokinetic properties and different pharmacological or toxicological effects [7].

Due to the chiral nature of the drug, it is felt necessary to develop a chiral LC method for the enantiomeric separation and accurate quantification of unwanted enantiomer ((*R*)-enantiomer) of Levetiracetam. This paper deals with method validation.

2. Experimental

2.1. Chemicals

Samples of Levetiracetam, (*R*)-enantiomer, *N*-(1-carbamoyl-propyl)-4-chloro-butyramide (Imp-1), and 1-ethyl-2-oxo-1-pyrrolidine acetic acid (Imp-2) were kindly supplied by Process Research Department of Dr. Reddy's Laboratories Limited, Hyderabad, India, and the chemical structures are given in Fig. 1. HPLC-grade hexane and isopropanol were purchased from Merck, Germany. Levetiracetam formulation (Keppra, 750 mg) was purchased from UCB Company.

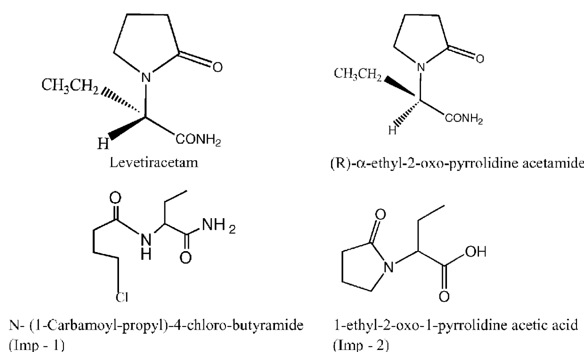

Fig. 1. Chemical structure of Levetiracetam, (*R*)-α-ethyl-2-oxo-pyrrolidine acetamide, Imp-1, and Imp-2.

2.2. Equipment

A Waters Alliance HPLC system equipped with 2695 separation module with inbuilt auto injector, 270852 thermostatic compartment, and 996 photo diode array detector was utilized for method development and validation in laboratory A. The second instrument, Waters LCM1 plus HPLC system equipped with 600 pump, 715 auto injector, 270852 thermostatic compartment, and 486 tunable absorbance detector, was utilized in ruggedness study in laboratory B. Millennium 32 chromatography manager software (Waters) was used for data acquisition and system suitability calculations. Photo diode array detector was used for determining peak purity.

2.3. Sample preparation

A stock solution of (*R*)-enantiomer (450 μg/ml) was prepared by dissolving the appropriate amount of the substance in isopropanol. The analyte concentration of Levetiracetam was fixed as 3 mg/ml. Working solutions of Levetiracetam and (*R*)-enantiomer were prepared in isopropanol.

Table 1
System-suitability report

Column name	Compound ($n = 3$)	k	R_S	N	T	α
Chiralcel OD-H	(R)-enantiomer	10.0	3.3	2100	1.6	
	Levetiracetam	13.7		2300	1.5	1.4
Chiralcel OJ-H	Levetiracetam	7.5	1.8	2860	2.2	
	(R)-enantiomer	8.9		3550	2.1	1.2
Chiralpak AD-H	(R)-enantiomer	8.1	7.9	4820	1.7	
	Levetiracetam	12.9		6450	1.6	1.6

$n = 3$ determinations. K—capacity factor; R_S—USP resolution; N—number of theoretical plates (USP tangent method); T—USP tailing factor; α—enantioselectivity. Marker used for dead time determination—Urasil (0.1 mg/ml in methanol). Eluent conditions: (1) column: Chiralpak AD-H (250 mm × 4.6 mm), 5 μm; (2) mobile phase: hexane:isopropanol (90:10); (3) flow rate: 1.0 ml/min; (4) column temperature: 25 °C. Dead time: 1.0 min.

2.4. Chromatographic conditions

The chromatographic conditions were optimized using an amylose-based chiral stationary-phase Chiralpak AD-H (250 mm × 4.6 mm, 5 μm, Daicel make), which was safeguarded with a 5-cm-long guard column. The mobile phase was hexane:isopropanol (90:10, v/v). The flow rate was set at 1.0 ml/min. The column was maintained at 25 °C, and the detection was carried out at a wavelength of 210 nm. The injection volume was 10 μl. Cellulose-based chiral stationary phases, Chiralcel OD-H and Chiralcel OJ-H (Daicel make), were also employed during method development. All calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

2.5. Validation of the method

2.5.1. System suitability test

System suitability test is an integral part of chromatographic methods and is used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed [8]. The system suitability test results of the chiral LC method on Chiralpak AD-H, Chiralcel OD-H, and Chiralcel OJ-H columns are presented in Table 1.

2.5.2. Selectivity

Selectivity is the ability of the method to measure the analyte response in the presence of its potential impurities. The selectivity of the developed chiral LC method was carried out in the presence of related impurities of Levetiracetam, namely Imp-1 and Imp-2. Method selectivity was also challenged by forced degradation of Levetiracetam sample under UV light (254 nm) and heat (60 °C) for 10 days. Content of (R)-enantiomer was checked in Levetiracetam sample exposed under light and heat on each day upto the study period. The exposed samples were tested for peak purity using photo diode array detector.

2.5.3. Precision

Precision of the method is the degree of agreement among the individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample. The allowed limit of (R)-enantiomer in Levetiracetam was fixed as 0.15%.

The precision of the method was checked by analyzing nine replicate samples of Levetiracetam (at the analyte concentration, i.e. 3 mg/ml) spiked with 0.15% (7500 ng/ml) of (R)-enantiomer and calculating the percentage relative standard deviation.

2.5.4. Limit of detection and limit of quantification of (R)-enantiomer

The limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantified as an exact value. The limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

LOD and LOQ for (R)-enantiomer were determined at a signal-to-noise ratio of 3 and 10 [9]. LOD and LOQ were achieved by injecting a series of dilute solutions of (R)-enantiomer.

The precision of the developed chiral method for (*R*)-enantiomer at limit of quantification was checked by analyzing six test solutions of (*R*)-enantiomer prepared at LOQ level and calculating the percentage relative standard deviation. The accuracy of the method was checked for (*R*)-enantiomer at LOQ level by analyzing three replicate samples of Levetiracetam (3 mg/ml) spiked with (*R*)-enantiomer at LOQ level and calculating the percentage recovery.

2.5.5. Linearity of (R)-enantiomer

The linearity of the method is a measure of how well a calibration plot of response versus concentration approximates a straight line.

Linearity was evaluated by determining nine working solutions of (*R*)-enantiomer ranging from 2250 (LOQ) to 9000 ng/ml (0.3%), (2250, 2700, 3375, 4050, 4500, 5625, 6765, 7875, and 9000 ng/ml), prepared in isopropanol from (*R*)-enantiomer stock solution.

The peak area and concentration of (*R*)-enantiomer were subjected to regression analysis to calculate calibration equation and correlation coefficient. Linearity was checked for three consecutive days in the same concentration range from the same stock solution. The percentage relative standard deviation of the slope and *Y*-intercept of the calibration curve was calculated.

2.5.6. Quantification of (R)-enantiomer in bulk sample

The Levetiracetam bulk sample, provided by Process Research Department of Dr. Reddy's Laboratories, showed the absence of distomer. Standard addition and recovery experiments were conducted to determine accuracy of the present method for the quantification of (*R*)-enantiomer in bulk drug samples.

The study was carried out in triplicate at 0.12, 0.15, and 0.18% of the Levetiracetam target analyte concentration. The recovery of (*R*)-enantiomer was calculated from the slope and *Y*-intercept of the calibration curve, drawn in the concentration range of 2250–9000 ng/ml (slope and *Y*-intercept values obtained in the linearity study).

2.5.7. Quantification of (R)-enantiomer in formulation

Keppra are film-coated tablets containing 250, 500, 750, and 1000 mg of Levetiracetam and are presented in PVC/ aluminum blisters. The excipients present in Keppra are maize starch, povidone K 30, colloidal anhydrous silica, and magnesium stearate. Five tablets of Keppra (750 mg) were finely ground using agate mortar and pestle. The ground material, which was equivalent to 1500 mg of the active pharmaceutical ingredient (Levetiracetam), was extracted into isopropanol in a 100 ml volumetric flask by vortex mixing followed by ultrasonication. The resultant mixture was filtered through 0.45 μm membrane filter. The filtrate was used as stock solution for preparing the accuracy test solutions. Five milliliters of the filtrate was taken in a 25 ml volumetric flask and made upto the volume with the isopropanol. This solution corresponds to analyte concentration of 3 mg/ml.

Standard addition and recovery experiments were conducted for (*R*)-enantiomer in Levetiracetam formulation (Keppra tablets) in triplicate at the same concentration levels as described in Section 2.5.6. The recovery of (*R*)-enantiomer was calculated from the slope and *Y*-intercept of the calibration curve obtained in Section 2.5.5.

2.5.8. Ruggedness

The ruggedness of a method was defined as degree of reproducibility of results obtained by analysis of the same sample under variety of normal test conditions such as different labs, different analysts, different instruments, and different lots of reagents. The standard addition and recovery experiments carried out for (*R*)-enantiomer in bulk drug samples at the concentration levels tested in Section 2.5.6 (laboratory A) were again carried out in laboratory B using a different instrument.

2.5.9. Robustness

The robustness of a method is the ability of the method to remain unaffected by small changes in parameters such as flow rate, mobile phase composition, and column temperature. To determine robustness of the method, experimental conditions were purposely altered and chromatographic resolution between Levetiracetam and (*R*)-enantiomer was evaluated.

The flow rate of the mobile phase was 1.0 ml/min. To study the effect of flow rate on the resolution of enantiomers, it was changed by 0.2 units from 0.8 to 1.2 ml/min. The effect of change in percent isopropanol on resolution was studied by varying from

−1 to +1% while the other mobile-phase components were held constant as stated in Section 2.4. The effect of column temperature on resolution was studied at 20 and 30 °C instead of 25 °C while the other mobile phase components were held constant as stated in Section 2.4.

2.5.10. Solution stability, mobile-phase stability, and stability of chiral stationary phase (Chiralpak AD-H)

Stability of Levetiracetam in solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for 2 days. Content of (*R*)-enantiomer was checked for 6-h interval upto the study period.

Mobile phase stability was carried out by evaluating the content of (*R*)-enantiomer in Levetiracetam sample solutions prepared freshly at 6-h interval for 2 days. Same mobile phase was used during the study period.

The standard mobile phase recommended to use for the chiral analysis of neutral samples in amylose-based chiral stationary phase, Chiralpak AD-H was hexane:isopropanol (90:10). The performance and stability of Chiralpak AD-H column used in the above study were checked with (*t*)-stilbene oxide after 2000 chromatographic injections and compared with column performance results supplied by the manufacturer (Daicel).

3. Results and discussion

3.1. Method development

The objective of this study was to separate the enantiomers of Levetiracetam and accurate quantification of the unrequired (*R*)-enantiomer. The racemic mixture was prepared by physical mixing the equal portions of Levetiracetam and (*R*)-enantiomer (0.5 g of each sample). A 0.5 mg/ml solution of racemic mixture prepared in isopropanol was used in the method development. The chiral impurity (other enantiomer) was found to be absent in the Levetiracetam and (*R*)-enantiomer samples selected for the study. Three different chiral columns were employed during method development, namely Chiralcel OD-H, Chiralpak AD-H, and Chiralcel OJ-H of Daicel. All the columns chosen were of 250 mm length, 4.6 mm internal diameter, and 5 μm particle size. The chiral stationary phase in Chiralpak AD-H, Chiralecl OD-H, and Chiralcel OJ-H columns was amylose tris (3,5-dimethylphenyl carbamate), cellulose tris(3,5-dimethylphenyl carbamate), and cellulose tris(4-methyl benzoate), respectively, coated on a silica gel. The mechanism of separation in direct chiral separation methods is the interaction of chiral stationary phase (CSP) with analyte enantiomers to form a short-lived, transient diastreomeric complexes [10]. The complexes are formed as a result of hydrogen bonding, dipole–dipole interactions, pi bonding, electrostatic interactions, and inclusion complexation. The relative binding strength of the diastereomeric complexes determines enantioselectivity and rate of elution of enantiomers. Baseline chromatographic separation was not achieved on a Chiralcel OJ-H column using the mobile-phase hexane:isopropanol (90:10), and eutomer was eluted prior to distomer. Very good separation was achieved on Chiralcel OD-H (resolution greater than 3) and Chiralcel AD-H (resolution greater than 7) columns using the same mobile phase and interestingly, distomer was eluted prior to eutomer. Since cellulose-based Chiralcel OD-H column had the same derivitization group (3,5-dimethylphenyl carbamate) as its amylose-based counterpart (Chiralpak AD-H), it showed same chiral recognition abilities for the enantiomers of Levetiracetam. The reversal of elution order of Levetiracetam enantiomers on Chiralcel OJ-H column could be due to the alteration of steric environment of the chiral cavities. Due to the better chromatographic results obtained on the Chiralpak AD-H column, the method validation was carried out on the same. Chiracel OD-H column can be used as an alternative column for the quantification of (*R*)-enantiomer.

In the optimized method, the typical retention times of (*R*)-enantiomer and Levetiracetam were 9.1 and 13.9 min, respectively. The enantiomeric separation of Levetiracetam on Chiralcel OD-H, Chiralcel OJ-H, and Chiralpak AD-H columns is shown in Fig. 2.

3.2. Validation results of the method

In the optimized chiral LC method, related impurities of Levetiracetam, namely Imp-1 and Imp-2, were well separated from Levetiracetam and (*R*)-enantiomer (Fig. 3). In the case of stress by UV light (254 nm)

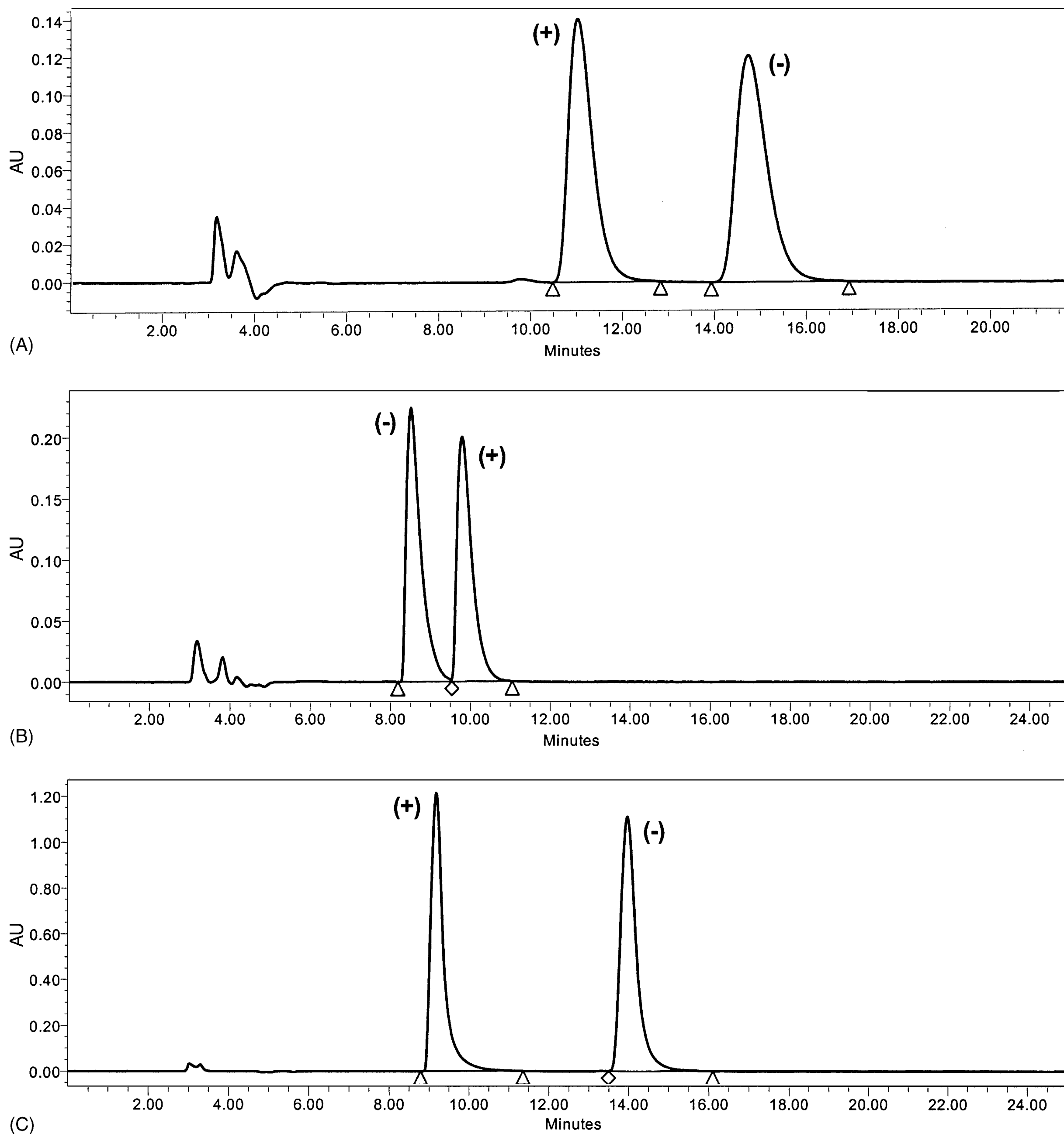

Fig. 2. Enantiomeric separation of racemic Levetiracetam on (A) Chiralcel OD-H, (B) Chiralcel OJ-H, and (C) Chiralpak AD-H columns; mobile phase composed of hexane:isopropanol (90:10, v/v); flow rate: 1.0 ml/min, UV-210 nm, column temperature: 25 °C.

and heat (60 °C), it was observed that rigorous stress of Levetiracetam sample did not cause any significant degradation and change in the (*R*)-enantiomer content for 10-day study period. The proposed chromatographic conditions were found to be selective to the Levetiracetam sample subjected to the applied stress conditions. Peak purity was obtained for Levetiracetam and (*R*)-enantiomer by overlay of the spectra

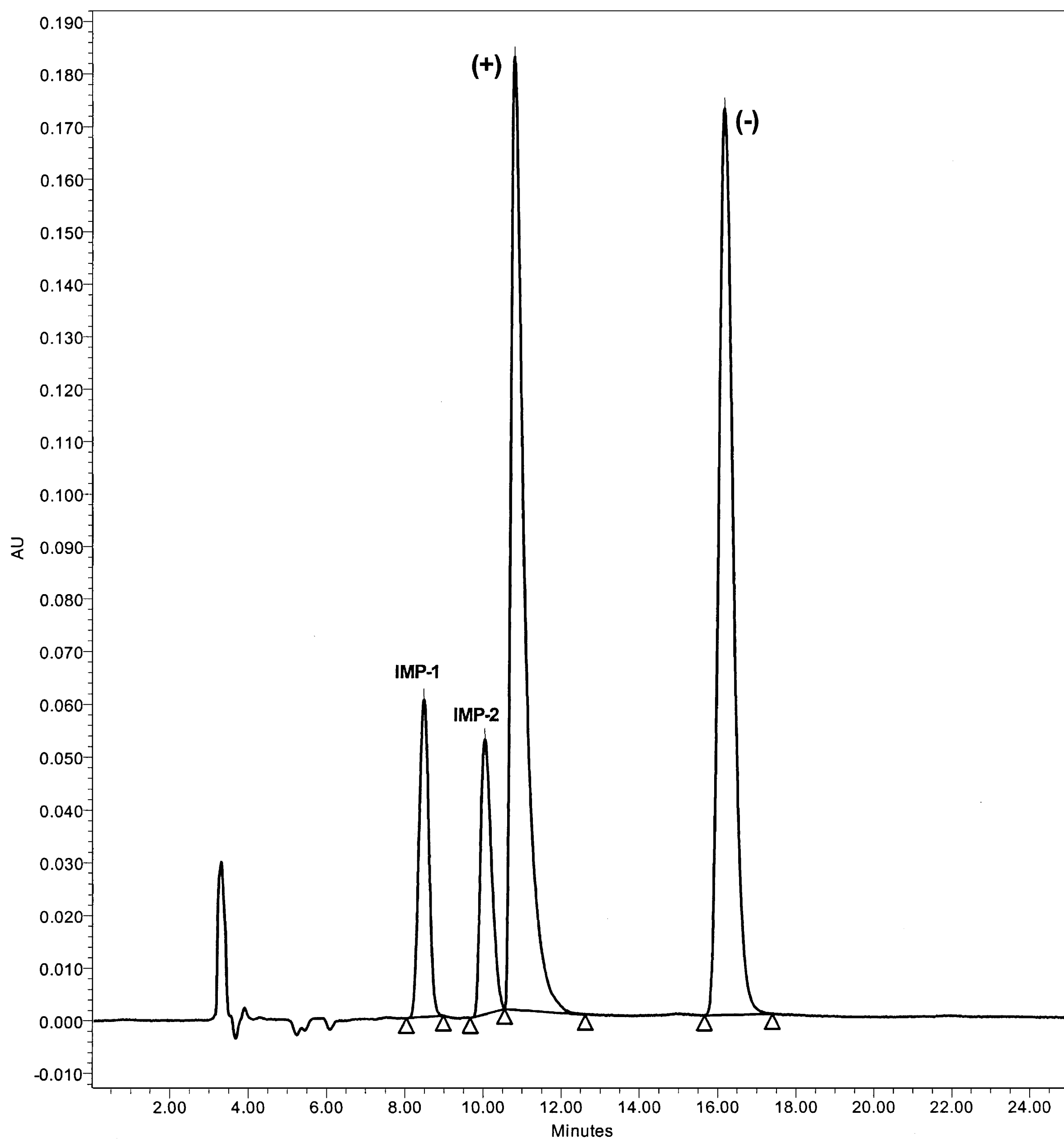

Fig. 3. Method selectivity in the presence of impurities, Imp-1 and Imp-2.

captured at the apex, up slope, and down slope using photo diode array detector, and no interference was noted for Levetiracetam and (*R*)-enantiomer in stress samples. Hence, the developed method is stability indicating and found to be selective.

In the precision study, the percentage relative standard deviation of analysis repeatability for Levetiracetanm and (*R*)-enantiomer was found to be 0.2 and 6%, respectively, indicating the good precision of the method.

Table 2
Precision results of (*R*)-enantiomer at LOQ level

Preparation	Area
1	21263
2	18545
3	21372
4	20675
5	18804
6	22659
	% R.S.D. 7.8

The LOD and LOQ concentrations were calculated to be 900 and 2250 ng/ml for (*R*)-enantiomer, when a signal-to-noise ratio of 3 and 10 were used as the criteria. The method precision for (*R*)-enantiomer at limit of quantification was less than 8% RSD (Table 2). The recovery of (*R*)-enantiomer at limit of quantification was 92% in the spiked Levetiracetam samples.

Under the optimized working conditions, standard calibration curve was constructed over the concentration range of 2250–9000 ng/ml for (*R*)-enantiomer, with the linear regression equation $y = 3362x + 290$ (correlation coefficient $R = 0.999$). The residual standard deviation of the calibration curve was less than 5%. Linearity was checked for (*R*)-enantiomer over the same concentration range for three consecutive days. The percentage relative standard deviation of the slope and *Y*-intercept of the calibration curve were 1.8 and 9, respectively. The results show that good correlation existed between the peak area and concentration of (*R*)-enantiomer.

In the quantification of (*R*)-enantiomer in bulk samples of Levetiracetam, standard addition and recovery experiments were conducted in triplicate at 0.12, 0.15, and 0.18% of analyte concentration. Recovery was calculated from slope and Y-intercept of the calibration curve obtained in linearity study and percentage recovery was ranged from 94.2 to 102.6 (Table 3).

Table 3
Recovery results of (*R*)-enantiomer in bulk drug sample

Added (ng)	Recovered (ng)	% Recovery	R.S.D. (%)
3605	3396	94.2	4.5
4510	4627	102.6	3.2
5405	5270	97.5	3.6

$n = 3$ determinations.

Table 4
Recovery results of (*R*)-enantiomer in commercial formulation

Added (ng)	Recovered (ng)	% Recovery	% R.S.D. (%)
3610	3375	93.5	4.5
4508	4307	95.5	3.8
5408	5630	104.1	4.0

$n = 3$ determinations.

Table 5
Ruggedness data of (*R*)-enantiomer in Laboratory B

Added (ng)	Recovered (ng)	% Recovery	% R.S.D.
3603	3416	94.8	4.0
4509	4608	102.2	3.5
5402	5321	98.5	3.9

$n = 3$ determinations.

(*R*)-enantiomer was found to be absent in the formulation samples of Levetiracetam (Keppra, 750 mg). Standard addition and recovery experiments were conducted to determine accuracy of the present method for the quantification of (*R*)-enantiomer in formulation samples. (*R*)-enantiomer was spiked to the extracted Levetiracetam solution (3 mg/ml) in triplicate at 0.12, 0.15, and 0.18% of analyte concentration. Recovery was calculated from the slope and *Y*-intercept of the calibration curve obtained in linearity study, and percentage recovery was ranged from 93.5 to 104.1 (Table 4). The unspiked and spiked chromatograms of the (*R*)-enantiomer at 0.15% of target analyte concentration in formulation sample are shown in Fig. 4.

Table 6
Robustness of the chiral LC method

Parameter	USP resolution between Levetiracetam and (*R*)-enantiomer
Flow rate (ml/min)	
0.8	9.0
1.0	8.1
1.2	7.2
Column temperature (°C)	
20	8.8
25	7.9
30	7.4
Isopropanol percentage in mobile phase	
9	8.7
10	7.8
11	7.4

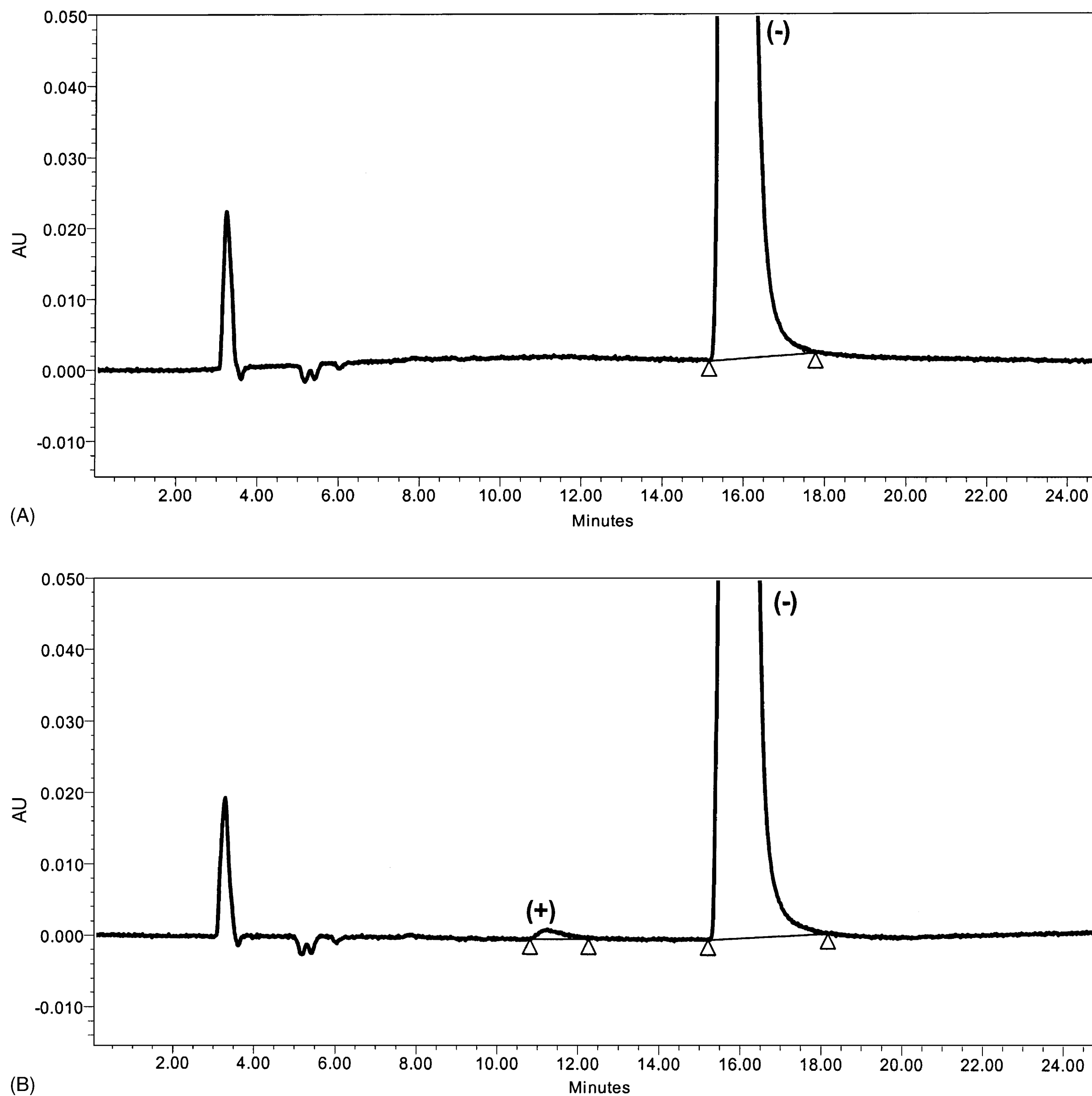

Fig. 4. LC chromatogram of (A) Levetiracetam formulation, (B) Levetiracetam formulation spiked with 0.15% (*R*)-enantiomer in the developed method.

Standard addition and recovery experiments were also conducted for (*R*)-enantiomer in bulk drug samples using a different system in laboratory B at the same concentration levels tested in laboratory A. The recovery results obtained in the laboratory B were well in agreement with the results obtained in laboratory A (Table 5). This confirms the ruggedness of the method.

The chromatographic resolution of the Levetiracetam and (*R*)-enantiomer peaks was used to evaluate the method robustness under modified conditions. Sufficient resolution for Levetiracetam and (*R*)-enantiomer was obtained under all separation conditions tested (Table 6), demonstrating sufficient robustness.

Table 7
Stability of chiral stationary phase (Chiralpak AD-H)

Retention time (min)	Theoretical plates (USP tangent method)	USP tailing	USP resolution
Column performance report (supplied by manufacturer)			
5.385	11140	1.28	
10.821	14710	1.07	11.2
Column performance report after 2000 chromatographic injections			
4.985	10580	1.42	
10.345	13954	1.22	10.5

Sample used: (*t*)-stilbene oxide.

No significant change in the (*R*)-enantiomer content was observed in Levetiracetam sample during solution stability and mobile phase stability experiments. Hence, Levetiracetam sample solution and mobile-phase are stable for at least 2 days.

Column performance results illustrate Chiralpak AD-H column used for quantification of (*R*)-enantiomer in Levetiracetam, is stable for at least 2000 chromatographic injections (Table 7).

4. Conclusion

A simple and accurate normal-phase chiral LC method was described for the enantiomeric separation of Levetiracetam. Chiralpak AD-H, an amylose-based chiral stationary phase, and Chiralcel OD-H, a cellulose based chiral stationary phase, were found to be selective for the enantiomers of Levetiracetam. Method validation was carried out using Chiralpak AD-H column due to the better chromatographic results achieved on the column. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the quantitative determination of chiral impurity ((*R*)-enantiomer) in pharmaceutical formulations and in-process materials.

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