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Determination of levetiracetam enantiomeric purity using HPLC-fluorescence detection coupled with a pre-column o-phthalaldehyde-*N*-acetyl-*L*-cysteine derivatization

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ABSTRACT: A simple and cost effective method was developed for enantiomeric separation of levetiracetam (LEV) and chiral impurity R-enantiomer in raw material and tablets. The method based on derivatization of racemate using o-phthalaldehyde and *N*-acetyl-*L*-cysteine in basic borate buffer. The diastereomeric isoindole derivatives were separated by HPLC efficiently using C18 column and ammonium acetate (20 mM, pH: 7.3): methanol (55:45) as mobile phase, and isocratic elution at a flow rate of 1.0 mL/min. The optimal fluorescence detection parameters were λex = 330 nm, and λem = 450 nm. The method was validated and applied to raw material and tablet samples. Enatiomeric resolution was achieved within 9 minutes, and the resolution factor was more than 2. Calibration curves were linear over the range 1-50 and 0.06-8.0 (µg/mL) for LEV and R-enantiomer, respectively. LOD and LOQ of R- enantiomer were 0.02, and 0.06 (µg/mL), respectively. The method was sensitive, accurate, and precise. The developed method had been applied successfully to determine R-enantiomer in LEV raw material and tablets and can be used in quality control laboratories.

KEYWORDS: Levetiracetam; *R*-enantiomer; *o*-phthalaldehyde; *N*-acetyl-*L*-cysteine; HPLC; fluorescence; validation.

1. INTRODUCTION

The novel antiepileptic drug levetiracetam (LEV) is administered as a single pure enantiomer (−)-(*S*)-ethyl-2-oxo-1-pyrrolidine acetamide rather than racemic mixture, since LEV is only responsible for anticonvulsant effect (Figure 1). It was approved by FDA in 2000 as adjunctive therapy for treatment of focal seizures, myoclonic seizure, and primary generalized seizure [1,2]. Levetiracetam (LEV) is characterized by efficacy, tolerability, and clinically insignificant drug interactions [3,4].

Many achiral methods were published to analyze LEV in dosage forms and biological matrices like plasma, saliva, and urine. It included HPLC-coupled with UV detection [5-8], spectrophotometric [9-11], spectrofluorimetric [12-14], HPTLC [15], GC-MS [16], and electrochemical [17] methods.

Chromatographic enantioselective separation and quantitation methods of levetiracetam and its *R*-enantiomer were described using different chiral stationary phases like chiral cyclodextrin [18], chiralpack AD-H [19], and chiral α1-acid glycoprotein [20]. In addition, a method of chiral cyclodextrin modified microemulsion electrokinetic chromatography (MEEKC) was reported [21].

There are different chiral chromatographic approaches – rather than chiral stationary phase- for resolution of enantiomers. Enantiomers may be separated by a derivatization reaction of the two enantiomers with a chiral derivatizing agent to form diastereomeric products, which are then separated by conventional HPLC. The latter approach has the advantage of improving the sensitivity due to chromophoric or fluorophoric properties of the derivatizing agent. In addition, the selectivity can be increased by the ability to optimize chromatographic conditions for a better resolution [22]. Chiral separation of LEV and its R-enantiomer based on diastereomeric derivatization and analysis on achiral stationary phase is still uninvestigated.

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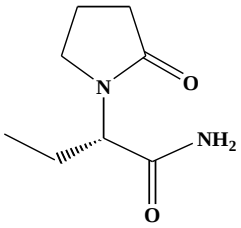

Figure 1. Chemical structure of levetiracetam.

o-Phthalaldehyde (OPA) is used in combination with a thiol to produce a fluorescent isoindole derivative of amines [23]. When a chiral thiol compound like *N*-acetyl-*L*-cysteine (NAC) is applied, then diastereomeric isoindole derivatives are produced (Figure 2). Separation of diastereomers is carried out using C18 columns. Columns of C18 type are inexpensive and mostly applied in routine quality control laboratories.

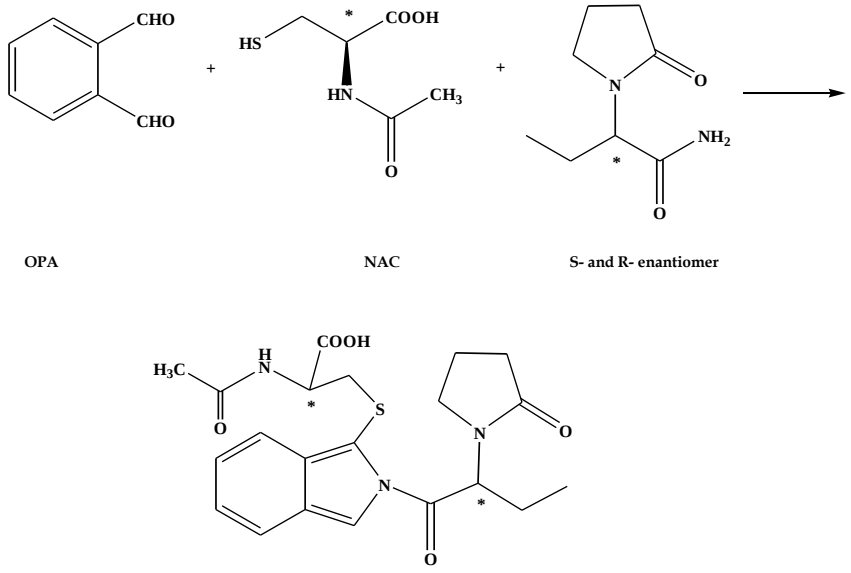

Figure 2. Derivatization reaction of enantiomers with o-phthalaldehyde (OPA) and *N*-acetyl-*L*-cysteine (NAC). Asterisks indicated chiral centers.

This study aimed to develop a sensitive, and inexpensive method to determine enantiomeric purity of (LEV) using a single step of pre-column derivatization reaction with OPA and NAC followed by RP-HPLC. The method is validated according to ICH guidelines [24]. Then it will be applied to samples of raw material and tablets.

2. RESULTS

2.1. Method development

The derivatization reaction of LEV and R-enantiomer mixture using OPA and NAC was optimized. Different parameters were investigated (Table 1). LEV racemate reacts completely with OPA and NAC in borate buffer pH 9.8 at room temperature within 5 minutes to produce isoindole derivatives as shown by the proposed mechanism in Figure 2. Derivatives were stable up to 3 hours (Peak area changed by less than 2%).

Resolution of diastereomers was evaluated using different C18 columns e.g. Shim-pack VP-ODS, Kromasil Eternity C18 column, Zorbax Eclipse XDB-C18 column, and LiChrospher RP-18 column. The optimal resolution was achieved using Shim-pack VP-ODS and ammonium acetate (20 mM, pH: 7.3): methanol (55:45) mobile phase in isocratic manner.

2.2. Validation

Validity of the developed method was examined according to ICH guidelines [24]. Different parameters were investigated like system suitability, linearity, range, specificity, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy.

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Table 1. Optimization of the derivatization reaction conditions.

Parameter	Value	Peak area[a]	Peak area[b]
OPA conc.(mM)	30	43114	52173
	50	42518	47013
	75	61263	60189
	100	63251	62175
	120	53114	57219
NAC conc. (mM)	15	51687	48996
	25	71053	67491
	35	50681	47834
	50	48897	43289
Borate buffer conc. (M)	0.05	48969	46935
	0.10	63861	60431
	0.15	50247	47596
	0.20	39893	45362
pH	9.0	49732	40367
	9.5	58632	47856
	9.8	65361	59869
	10.0	61783	56143
	10.5	57869	51321
	11.0	56115	46896

[a]: Average peak area of LEV-derivative (Two determinations).
[b]: Average peak area of R-enantiomer derivative (Two determinations).

2.2.1. System suitability

System suitability is a fundamental part of chromatographic system. It is applied to prove that reproducibility and peak resolution are sufficient for the analytical purpose [25].The derivative of R-enantiomer was well resolved from that of LEV with a resolution factor more than 2 (Figure 3). The retention times of derivatives for R-isomer and LEV were 6.0, and 8.8 minutes, respectively. The data of system suitability at optimized conditions are given in Table 2.

2.2.2. Linearity and range

The peak area was plotted against concentration over the range of 0.06 - 8.00 (µg/mL) for R-enantiomer and 1- 50 (µg/mL) for LEV. The linear regression equation of LEV was Y = 30.53 X – 1683 and for R-enantiomer was Y = 34.69 X – 1897, where Y is average peak area, X is the concentration (µg/mL), with correlation coefficients of 0.9997 and 0.9991, respectively.

2.2.3. Specificity

The specificity is the ability of a method to measure accurately an analyte in the presence of interference like excipients, degradation products, synthetic precursors, and potential matrix related impurities. The developed method was tested by taking into account the possibility of interference coming from the widely used excipients in tablet formulation, such as talc, lactose, starch, mannitol and magnesium stearate.No interferences were observed at retention times of LEV and R-isomer derivative from other related substances (Figure 3).

Table 2. System suitability parameters.

Compound	R_t[a], min.	R_s[b]	N[c]	T[d]
R-isomer	6.0	-	2158	1.21
LEV	8.8	2.4	2230	1.10

[a]: Retention time
[b]: Resolution factor.
[c]: Number of theoretical plates.
[d]: Tailing factor.

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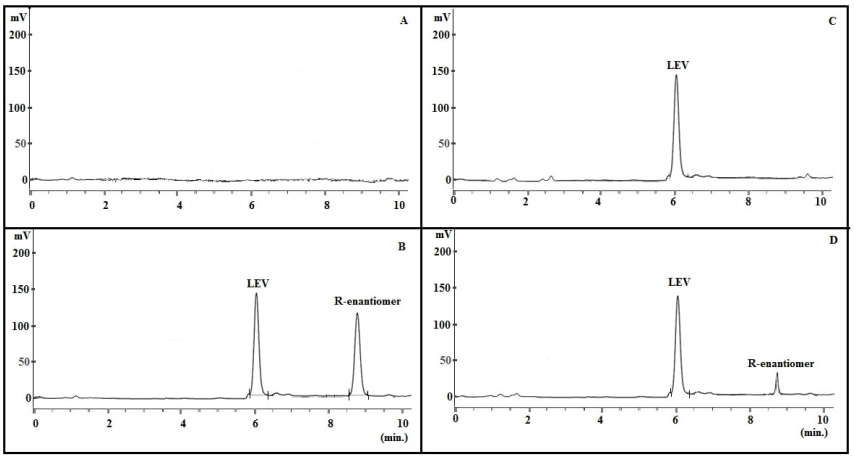

Figure 3. Chromatograms of isoindole derivatives of LEV (Rt: 6.0 min), and R-enantiomer (Rt: 8.8 min). Conditions: Mobile phase ammonium acetate (20 mM, pH: 7.3): methanol (55:45); flow rate 1.0 mL/min; C18 stationary phase; fluorescence detection. A: Blank, B: Mixture of derivatives of standard LEV and R enantiomer (5µg/mL), C: LEV (5µg/mL) spiked with excipients, D: LEV (5µg/mL) and R-enantiomer (0.07 µg/mL) spiked with excipients.

2.2.4. Limit of detection and limit of quantitation

Limit of detection LOD and limit of quantitation LOQ represent the sensitivity performance. LOD was 0.02 (µg/mL) and LOQ was 0.06 (µg/mL) for R-isomer. For LEV the LOD and LOQ were 0.3 and 1.0 (µg/mL), respectively.

2.2.5. Accuracy

Accuracy is a measure of how close the experimental value to the true value. It was performed by spiking LEV and R-enantiomer at three different levels with tablet formulation excipients and the percent recovery was evaluated. The percent recovery values of R-enantiomer ranged from 95.50 to 103.97% indicating good accuracy. The percent SD was ≤ 5% for 3 replicates (Table 3). For LEV the percent recovery ranged from 99.06 to 101.57%. Recovery studies fulfilled the acceptance criteria [24].

2.2.6. Precision

Precision is the measure of how close the data values to each other for a number of measurements under the same analytical conditions [25]. Regarding intra- and inter-day precision, the percent of relative standard deviation %RSD was evaluated for five and three replicates, respectively. Values of percent RSD were ≤ 2% for LEV and ≤ 3.8% for R-enantiomer (Table 4). These values indicated that analysis has adequate precision [24-26].

Table 3. Recovery study of the developed method (n = 3).

R-enantiomer		LEV	
Conc. (µg/mL)	**% Recovery (Mean ± SD)**	**Conc. (µg/mL)**	**% Recovery (Mean ± SD)**
0.07	95.50 ± 4.21	5	100.13 ± 0.91
0.4	98.97 ± 3.11	30	99.06 ± 1.04
7.0	103.97 ± 2.25	50	101.57 ± 1.13

Table 4. Intra- and inter-day precision of the developed method.

R-enantiomer			LEV		
Conc. µg/mL)	**%RSD**		**Conc. (µg/mL)**	**%RSD**	
	ntra-day (n = 5)	**Inter-day (n = 3)**		**Intra-day (n = 5)**	**Inter-day (n = 3)**
0.07	2.65	3.8	5	1.85	1.81
0.4	2.31	2.01	30	1.59	1.34
7.0	1.76	1.89	50	1.13	0.86

2.2.7. Robustness

Robustness tests examine the effect of small changes in chromatographic conditions e.g. flow rate, column temperature, and mobile phase composition on resolution of peaks. The results are listed in Table 5. Chromatographic parameters were deliberately altered, and the resolution was checked between peaks of LEV and R-enantiomers. The resolution factor was affected insignificantly with minor changes in chromatographic conditions, which indicated good robustness of the method [24-26].

Table 5. Robustness data of the developed method.

Parameter	Variation	Resolution factor
Flow rate (mL/min)	0.8	2.5
	1.2	2.3
Temperature (°C)	20	2.3
	30	2.1
Percent methanol in mobile phase	43	2.3
	47	2.2

2.3. Analysis of samples

Raw material and commercial LEV tablets were analyzed by proposed method. The results are given in Table 6. R-enantiomer was not detected, and the label claim percentage of LEV was 99.02% - 101.21%.

Table 6. Results of sample analysis by the developed method (Mean ± SD, n = 3).

Sample	%LEV (w/w)	R-enantiomer
Raw material	99.02 ± 0.31	Not detected
Setanorm tablet	101.21 ± 1.07	Not detected
Soprotam tablet	99.61 ± 1.43	Not detected
Levepsy tablet	100.68 ± 0.59	Not detected

3. DISCUSSION

Direct chiral enantiomeric separation of LEV racemate was studied using chiral stationary phases. However, indirect separation through building of diastereomers is still uninvestigated. The indirect method has several advantages such as enhanced sensitivity of derivatization products, commercial availability of a large number of chiral derivatizing agents, low cost of achiral column, well-established derivatization reactions leading to diastereomeric products with excellent separation, and a wide choice of chromatographic conditions. However, indirect chiral separation via derivatization reaction has some principal disadvantages like time consumption during the reaction, risk of racemization, interference liability of excess reagents and side products with diastereomeric separation, and the possibility of resolution kinetic [27].

Derivatization using OPA and NAC in basic medium was applied for resolution of aminoacids [28-30]. OPA – NAC reagent was chosen since it is an economical reagent, produces stable diastereomers, and easy to operate. A mixture of LEV and R-enantiomer was used for the pre-column derivatization reaction at appropriate concentration of the analytical purpose. In a basic borate buffer (pH 9.8) LEV and R-enantiomer reacts essentially with the non-fluorescent OPA in the presence of a nucleophilic thiol compound (NAC) to produce fluorescent isoindole derivatives. The reaction proceeded rapidly at room temperature. The optimal peak area of both isomers was achieved when the molar ratio of OPA: NAC was 4:1. A significant excess of OPA was necessary to get the derivatization reaction complete, the mole ratio of R-enantiomer: OPA was 1:40. Higher concentrations of OPA had not improved the reaction. In addition, excess OPA can influence the stability of derivatives [31].

The proposed mechanism of the derivatization reaction is illustrated in Figure 2. The reaction offers numerous advantages such as simplicity of reaction conditions, rapidly proceeding reaction, efficient derivatization of the two enantiomers, and the high stability of products. In addition, excess reagents of OPA or NAC has no influence on fluorimetric measurements [30,31].

OPA-NAC derivatization reaction produced a highly fluorescent and UV-absorbing isoindole product. Fluorimetric detection was chosen in this study due to the high sensitivity [30-32]. The resolution of isoindole diastereomeric derivatives was adequate on C18 column Shim-pack VP-ODS. The chromatographic conditions

were simple isocratic elution using mobile phase of ammonium acetate and methanol. The elution of diastereomers was relatively short. The reported resolution factor was more than 2, which met the desired requirements of guidelines for chiral purity (> 1.7) [24-26]. In addition, the resolution factor obtained by the developed method was comparable with previously reported factors based on chiral stationary phases for LEV separation form R-enantiomer [19,20]. To evaluate the acceptability of the developed chromatographic conditions system suitability test was performed and the results are given in Table 2. The tailing factor was less than 1.5, which showed sharp and integral peaks and met standard limits [24-26]. As a sequence of good agreement of suitability test with requirements, validation parameters were examined.

Validation parameters were performed according to ICH and USP guidelines for impurity in bulk and finished pharmaceutical products [24,26]. The obtained results showed that the developed method had fulfilled the different requirements of validation parameters. It was accurate, specific, precise, and robust. The content of LEV was assayed successfully in samples of raw material and tablet and the results complied with USP monographs [33,34].

The developed method was compared with published methods for enantiomeric resolution of LEV and R-enantiomer. Parameters of comparison are listed in Table 7. The enantiomeric separation achieved by the pre-column OPA-NAC derivatization and RP-HPLC was satisfactory for the analytical purpose. Resolution factor recorded in the developed method was comparable with reported value for a method based on chiral α1 acid glycoprotein [20]. In comparison to chiral stationary phase, RP-HPLC technique is convenient, cost-effective, and the development of chromatographic conditions is simple. In addition, specific chiral stationary phases and toxic organic solvent-based mobile phases were avoided. The derivatization step of LEV and R-enantiomers using OPA/NAC prior to chromatography improved the sensitivity. A LOQ (0.06 µg/mL) was achieved for R-enantiomer, which was more sensitive than a previously reported limit (0.4 µg/mL) as shown in Table 7. Assaying chiral purity of LEV was achieved by a cost effective, rapid and sensitive HPLC method coupled with a pre-column derivatization.

Table 7. Important parameters of the enantiomeric separation of LEV and R-enantiomer by the developed method and liquid chromatographic methods.

Method	Stationary Phase	Rs	Run time (min.)	Range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Ref.
HPLC-UV	chiral-α1-acid glycoprotein	2.0	4.0	0.4-20	0.1	0.4	20
	Chiralcel OD-H	3.3	18	n.a	n.a	n.a	
	Chiralcel OJ-H	1.8	12	n.a	n.a	n.a	19
	Chiralpack AD-H	7.9	18	2.25-9.0	0.9	2.25	
Developed method	C18-Reverse phase	2.4	9.0	0.06-8.0	0.02	0.06	-

n.a: Not available.

4. CONCLUSION

A simple and sensitive method was developed and validated for determination of levetiracetam chiral purity and assaying R-enantiomer in raw material and tablets. The method based on a pre-column derivatization using OPA and NAC and RP-HPLC. Enantiomeric separation was achieved effectively with 9.0 minutes using inexpensive stationary phases and water based mobile phase. The method was applied successfully for analysis of R enantiomer in raw material and tablets of LEV.

5. MATERIALS AND METHODS

5.1. Chemicals and reagents

OPA and NAC, boric acid, sodium hydroxide and ammonium acetate of p.a. grade were purchased from Sigma–Aldrich (Germany). Methanol and water HPLC-grade (Merck, Germany) were used. LEV and R-enantiomer were gift samples from a local company Jerusalem Pharmaceuticals Co., West Bank, Palestine.

5.2. Chromatographic system

HPLC apparatus is Prominence LC-20AB solvent delivery unit equipped with fluorescence detector (RF-20A) and vacuum degasser (DUG-20A5R). LabSolution Software was used for data acquisition and processing from Shimadzu (Kyoto, Japan). Chromatographic system consists of Shim-pack® VP-ODS (250 × 4.6 mm, 5 µm particle size) from Shimadzu (Kyoto, Japan) as stationary phase, and 20 mM ammonium acetate (pH: 7.3): methanol (55:45) as mobile phase. The elution followed at 1.0 ml/min in isocratic manner at room temperature. The fluorimetric detector was set to an excitation wavelength of 330 nm and an emission wavelength of 450 nm. The injection volume was 20 µL.

5.3. Pre-column derivatization

5.3.1. Preparation of reagents

Derivatization reagents were prepared by dissolving separately 135 mg OPA (100 mM), and 40 mg NAC (25 mM) in 10 mL methanol. Reagents were stable for 4 weeks in dark place at 4 ºC. Borate buffer (0.1 M, pH 9.8 ± 0.05) was prepared by dissolving 0.6180 g of boric acid in ca. 80 mL of HPLC-water, adjusted pH using 2.0 M NaOH and made up to 100 mL. Standard solution of LEV and R-enantiomer were prepared at concentration 1 mg/mL in water and kept at 4 ºC. Working standard solutions were prepared by diluting appropriate volumes of standard solution with water to produce solutions ranged from 0.06-8.00, and 1-50 (µg/mL) for R-isomer, and LEV, respectively. All solutions were brought to room temperature before use.

5.3.2. Derivatization reaction

For derivatization the following reagents were pipetted in the following order to 100 µL of working standard solution 100 µL OPA, 100 µL NAC, 1 mL borate buffer, vortexed for 30 seconds and stirred for 5 minutes at room temperature. 20 µL of the mixture were injected onto the column. To optimize the derivatization reaction the following factors were investigated: pH (9.0-11.0), borate buffer concentration (0.05-0.2 M), OPA concentration (30-120 mM), and NAC (15-50 mM). Stability of isoindole derivative was examined by following peak area at interval times 0.5-12 h after mixing the components for (5 µg/mL) of LEV and R-enantiomer.

5.4. Validation

5.4.1. system suitability

System suitability was verified by analyzing pure enantiomers in five replicates and determining the sequence of elution, peak symmetry, column efficiency, and resolution factor.

5.4.2. Linearity and range

For Linearity five concentrations within the range 0.06-8.00 (µg/mL) for R-enantiomer and 1-50 (µg/mL) were prepared for linearity testing. Specificity was examined by spiking enantiomers with excipients used in drug formulation and quantified by the proposed method.

5.4.3. LOD and LOQ

LOD and LOQ were determined for R-isomer - since it present at lower concentration- at a Signal: Noise 3:1, and 10:1, respectively.

5.4.4. Accuracy and precision

For accuracy three replicate for 0.07, 0.4, 7.0 (µg/mL) of R-enantiomer and for LEV at 5.0, 30, 50 (µg/mL) mixed with formulation excipients were analyzed, and the percent recovery was calculated. Intra-day and inter-day precision were evaluated by calculating percent relative standard deviation (%RSD) for 5 replicates for three concentration within a day and on three days within a week, respectively.

5.4.5. Robustness

Robustness investigates the effect of minor changes in operational parameters of an analytical method on the results. For this purpose the flow rate, column temprature, and percent methanol in mobile phases were slightly modified and the resolution factor was evaluated in each case. Flow rate was changed by 0.2 unit, and the column temprature was adjusted at 20 ºC and 30 ºC. The percent methanol in mobile phase was examined at 43% and 47%. One factor is only changed, while others remain constant.

5.5. Preparation of samples

Twenty tablets of LEV were weighed and finely powdered. A portion of the powder equivalent to about 100 mg of LEV was weighed and dissolved with water in 100 mL volumetric flask and filtered through a cellulose membrane syringe filter. Dilution was made with water to produce a concentration within the calibration range. Diluted sample solution was subjected to derivatization reaction and chromatography. For raw material of LEV, the same procedure was applied.

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