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Short communication

# A validated chiral liquid chromatographic method for the enantiomeric separation of safinamide mesilate, a new anti-Parkinson drug

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ABSTRACT

A enantioselective reversed-phase high performance liquid chromatographic method was developed for the enantiomeric resolution of safinamide mesilate, 2(*S*)-[4-(3-fluorobenzyloxy)benzylamino] propionamide methanesulfonate, a neuroprotectant with antiparkinsonian and anticonvulsant activity for the treatment of Parkinson disease. The enantiomers of safinamide mesilate were baseline resolved on a Chiralcel OD-RH (150 mm × 4.6 mm, 5 μm) column using a mobile phase system containing 300 mM sodium di-hydrogen phosphate buffer (pH 3.0):methanol:acetonitrile (65:25:10, v/v/v). The resolution between the enantiomers was not less than 3.0. The pH value of buffer solution in the mobile phase has played a key role in enhancing chromatographic efficiency and resolution between the enantiomers. The developed method was validated and proved to be robust. The limit of detection and limit of quantification of (*R*)-enantiomer were found to be 15 and 50 ng/mL, respectively, for 20 μL injection volume. The percentage recovery of (*R*)-enantiomer was ranged from 94.2 to 103.7 in bulk drug samples of safinamide mesilate. The sample solution and mobile phase were found to be stable at least for 48 h. The final optimized method was successfully applied to separate (*R*)-enantiomer from safinamide mesilate and was proven to be reproducible and accurate for the quantitative determination of (*R*)-enantiomer in bulk drugs.



## 1. Introduction

Since the discovery of difference between thalidomide enantiomers in pharmacological and toxicologic actions, discrimination of optical isomers has been one of the major subjects in the field of pharmacy, because optical purities of substrates with asymmetries are critical for the evaluation of their biological activities [1]. Enantiomers of racemic drugs often show different behaviors in pharmacological action and metabolic process. Often one enatiomer is active while the other can be non-active, poorly active or toxic. The pharmaceutical industry has raised its emphasis on the generation of enantiomerically pure compounds before undertaking pharmacokinetic, metabolic, physiological and toxicological evaluation in the search for drugs with greater therapeutic benefits and low toxicity [2,3].

High performance liquid chromatography (HPLC) is playing a more and more important role for the resolution of drug enantiomers in the field of pharmaceutical industry [4]. However, the development of the methods for the quantitative analysis of chiral compounds and for the assessment of enantiomeric purity is extremely challenging, because the same physical and chemical properties of the two enantiomers make discriminating and separating them very difficult. Recently, many chromatographic methods have been reported describing the use of chiral stationary phases in conjunction with HPLC, as ways to separate and thereby individually quantitate the enantiomers of an enantiomeric pair [5–8].

Safinamide mesilate, 2(*S*)-[4-(3-fluorobenzyloxy)benzylamino] propionamide methanesulfonate, as a neuroprotectant with antiparkinsonian and anticonvulsant activity for the treatment of Parkinson disease, is a novel sodium and calcium channel blocker endowed with selective and reversible inhibition of monoamino oxidase type B (MAO-B) [9].

Safinamide mesilate has been evaluated in preclinical and clinical studies [10–12]. It is prepared from (*S*)-alaninamide as a single enantiomer [13], while the (*R*)-enantiomer is the undesired enantiomer, which can be present as a chiral impurity. In the literature, three methods were reported for the bioassay of safinamide in biological fluids of humans and various animals, using the tandem mass spectrometry (LC–MS–MS) system and high performance liquid chromatography with fluorimetric detection (HPLC-FD) [14]. However, there is no reference for the enantiomeric separation of safinamide mesilate enantiomers in bulk drugs using HPLC. So it is essential to establish an effective method to analyze the

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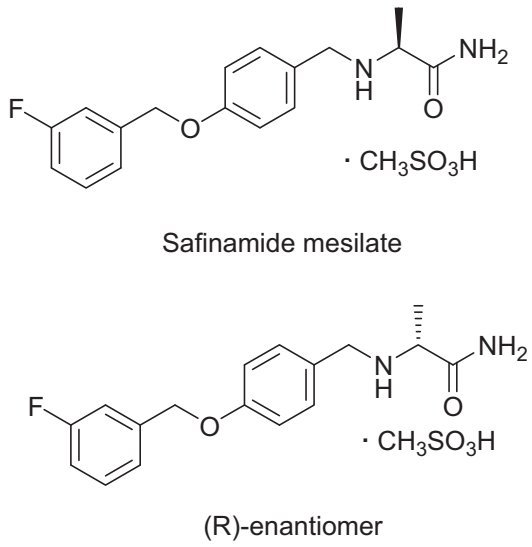


**Fig. 1.** Chemical structures of safinamide mesilate and (*R*)-enantiomer.

enantiomers of safinamide mesilate, and our report can be an element task for further researches. The chemical structures of safinamide mesilate and (*R*)-enantiomer are shown in Fig. 1, and (*R*)-enantiomer from safinamide mesilate may be at low level because of little (*R*)-alaninamide existing in starting material or racemization in synthesis.

This paper describes a chiral HPLC method for the enantiomeric separation of safinamide mesilate enantiomers using a modified cellulose based chiral stationary phase, Chiralpak OD-RH. The aim of this work was to optimize the chromatographic conditions in terms of temperature, pH value of buffer solution and mobile phase composition in order to separate and identify the enantiomers of safinamide mesilate. The developed HPLC method was reproducible and accurate for the quantitative determination of (*R*)-enantiomer in safinamide mesilate.

## 2. Experimental

### 2.1. Chemicals

Safinamide mesilate was kindly provided by Shijiazhaung Pharma Group NBP Pharmaceutical Ltd. (Shijiazhaung, China). (*R*)-enantiomer (the undesired enantiomer) was prepared in our laboratory. HPLC-grade methanol and acetonitrile were purchased from TEDIA (USA). Sodium di-hydrogen phosphate dihydrate, ortho-phosphoric acid and sodium hydroxide were purchased from Alfa Aesar (Tianjin, China). HPLC water from Heal Force system (Beijing, China) was used.

### 2.2. Columns

Preliminary column screening involved protein-based Chiral HPLC Columns, namely: Chiral AGP (150 mm × 4.0 mm, 5 μm), Chiral HSA (150 mm × 4.0 mm, 5 μm), Chiral CBH (150 mm × 4.0 mm, 5 μm) of Chromtech and then Chiralpak AD-RH (150 mm × 4.6 mm, 5 μm), Chiralcel OJ-RH (150 mm × 4.6 mm, 5 μm) of Daicel were also employed. The column used in the major method development activities was a modified cellulose based chiral column: Chiralcel OD-RH (150 mm × 4.6 mm, 5 μm, Daicel, Japan) column.

### 2.3. Chromatography

Chromatography was carried out using Agilent Technologies 1200 series instrument (USA) equipped with column oven, UV detector, and the data was processed using a computer program (Chemstation). The chromatographic conditions were optimized using a chiral stationary phase, Chiralcel OD-RH column (150 mm × 4.6 mm, 5 μm, Daicel, Japan). The isocratic mobile phase composition was a mixture of 300 mM sodium di-hydrogen phosphate buffer (pH 3.0):methanol:acetonitrile (65:25:10, v/v/v), which was pumped at a flow rate of 0.5 mL/min. The temperature of the column was maintained at 25 °C, and the eluant was monitored at a wavelength of 224 nm. The injection volume was 20 μL.

### 2.4. Sample preparation

Stock solutions of safinamide mesilate (1.0 mg/mL) and (*R*)-enantiomer (1.0 mg/mL) were prepared by dissolving the appropriate amount of the substances in methanol. The analyte concentration of safinamide mesilate was fixed as 50 μg/mL. Safinamide solutions spiked with low levels of (*R*)-enantiomer were prepared by transferring calculated amount of undesired enantiomer stock solution with pipette into the calculated amount of safinamide mesilate stock solution, and then the solution was added to volume with mobile phase and mixed well.

### 2.5. Validation of the method

#### 2.5.1. Method reproducibility

Method reproducibility was determined by measuring repeatability and intermediate precision of retention times and peak areas for each enantiomer. The repeatability of the method was determined by analyzing six replicate injections containing safinamide mesilate (50 μg/mL) spiked with (*R*)-enantiomer (0.6%, 300 ng/mL). The intermediate precision was determined over 3 days by performing six successive injections each day.

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

The limit of detection (LOD), defined as lowest concentration of analyte that can be clearly detected above the base line signal, is estimated as 3 times the signal to noise ratio [15]. The limit of quantitation (LOQ), defined as lowest concentration of analyte that can be quantified with suitable precision and accuracy, is estimated as 10 times the signal to noise ratio [15]. 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 was checked by analyzing six test solutions of (*R*)-enantiomer prepared at the LOQ level and calculating the percentage relative standard deviation of area.

#### 2.5.3. Linearity of (R)-enantiomer

Detector response linearity was assessed by preparing six calibration sample solutions of (*R*)-enantiomer covering from 50 ng/mL (LOQ) to 600 ng/mL (50, 100, 150, 250, 400 and 600 ng/mL) in mobile phase. Regression curve was obtained by plotting peak area versus concentration, using the least squares method. 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.

#### 2.5.4. Quantification of (R)-enantiomer in bulk sample

The safinamide mesilate bulk sample, provided by Shijiazhaung Pharma Group NBP Pharmaceutical Ltd., showed the absence of

(*R*)-enantiomer. Standard addition and recovery experiments were conducted to determine the present method for the quantification of (*R*)-enantiomer in bulk drug samples. The study was carried out in triplicate at 0.4, 0.6 and 0.8% of the safinamide mesilate target analyte concentration. The recovery of (*R*)-enantiomer was calculated from the slope and *Y*-intercept of the calibration curve obtained.

*2.5.5. Robustness*

The robustness of a method is the ability of the method to remain unaffected by small changes in parameters such as flow rate, pH value of buffer solution, mobile phase composition and column temperature. Chromatographic resolution between safinamide mesilate and (*R*)-enantiomer was used to evaluate the robustness of the method.

The flow rate of the mobile phase was 0.5 mL/min. To study the effect of flow rate on the resolution of enantiomers, it was changed at the pace of 0.05 units from 0.45 to 0.55 mL/min. The effect of a minor increase or decrease in acetonitrile volume fraction (±1%), pH value of buffer solution (±0.1) and column temperature at 20 and 30 °C instead of 25 °C on resolution were also studied while the other chromatographic conditions were same as described in Section 2.3.

*2.5.6. Solution stability and mobile phase stability*

Stability of safinamide mesilate 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. The content of (*R*)-enantiomer was checked at 6 h interval up to the study period. Mobile phase stability was carried out by evaluating the content of (*R*)-enantiomer in safinamide mesilate sample solutions prepared freshly at 6 h interval for 2 days. Same mobile phase was used in the experiment.

## 3. Results and discussion

### 3.1. Optimization of chromatographic conditions

Racemic mixture solution of safinamide mesilate and (*R*)-enantiomer (25 μg/mL each) prepared in mobile phase was used in the method establishment. To develop a rugged and suitable HPLC method for the separation of the two enantiomers, different stationary phases and mobile phases were employed. Initial screening of chiral column was carried out by several Chiral HPLC Column suppliers. Various protein-based Chiral HPLC Columns, namely: Chiral AGP, Chiral HAS and Chiral CBH of Chromtech, and then Chiralcel OJ-RH, Chiralpak AD-RH and Chiralcel OD-RH of Diacel, were also employed. Only Chiralpak AD-RH [amylose tris-(3,5-dimethylphenylcarbamate)] and Chiralcel OD-RH [cellulose tris-(3,5-dimethylphenylcarbamate)] columns provided selectivity between safinamide peak and the undesired enantiomer peak using a mobile phase consisting of 100 mM sodium di-hydrogen phosphate buffer (pH 4.0):methanol (70:30, v/v), but the retention times of safinamide and (*R*)-enantiomer were both longer than 30 min and the peaks were broad.

We continued to select the best mobile phases that would give optimum resolution and selectivity for the two enantiomers. To shorten the retention times of the two enantiomers, right amount of acetonitrile was added in the mobile phase. Then pH value and concentration of the phosphate buffer solution in mobile phase on Chiralcel OD-RH and Chiralpak AD-RH columns were optimized to enhance the chromatographic efficiency and resolution between the enantiomers (Tables 1 and 2). Good separation was achieved on Chiralcel OD-RH (resolution greater than 3.0) and Chiralpak AD-RH (resolution greater than 1.5) columns using 300 mM sodium di-hydrogen phosphate buffer (pH 3.0):methanol:acetonitrile (65:25:10, v/v/v) as mobile phase. The peaks of the two enantiomers became broad and the resolution fell obviously with high pH value of phosphate buffer. It is clear from the data that lower pH value and higher concentration of phosphate buffer can enhance the chromatographic efficiency and resolution. However, for long working life of the columns, we chose 300 mM sodium di-hydrogen phosphate buffer (pH 3.0) as final aqueous phase. Due to the better chromatographic results obtained on Chiralcel OD-RH column, further method optimization and quantification of (*R*)-enantiomer were carried out on this column.

Based on the data obtained from method development and optimization activities, Chiralcel OD-RH (150 mm × 4.6 mm, 5 μm) column with mobile phase of 300 mM sodium di-hydrogen phosphate buffer (pH 3.0):methanol:acetonitrile (65:25:10, v/v/v) was selected for the final method. The flow rate of the experiment method was 0.5 mL/min with an injection volume of 20 μL. The column temperature was 25 °C, and the detection wavelength was 224 nm. Under these conditions, the two enantiomers were separated well and the peak of (*R*)-enantiomer eluted before the peak of safinamide. In the optimized method, the typical retention times of (*R*)-enantiomer and safinamide were approximately 9.96 and

**Table 1**
The selectivity of safinamide mesilate enantiomers on Chiralpak OD-RH column with different pH values of burrer salt solutions in modifiers [sodium di-hydrogen phosphate buffer:methanol:acetonitrile (65:25:10, v/v/v)] at a flow rate of 0.5 mL/min and 25 °C.

| Burrer salt solution | $k'_1$ | $k'_2$ | Rs | $\alpha$ |
|---|---|---|---|---|
| pH value of 100 mM sodium di-hydrogen phosphate buffer | | | | |
| 3.0 | 3.34 | 3.71 | 2.62 | 1.11 |
| 3.5 | 3.45 | 3.82 | 2.21 | 1.11 |
| 4.0 | 3.96 | 4.31 | 1.64 | 1.09 |
| 5.0 | 5.47 | 5.82 | 1.07 | 1.06 |
| pH value of 200 mM sodium di-hydrogen phosphate buffer | | | | |
| 3.0 | 3.12 | 3.58 | 3.06 | 1.15 |
| 3.5 | 3.37 | 3.81 | 2.72 | 1.13 |
| 4.0 | 3.71 | 4.24 | 2.05 | 1.14 |
| 5.0 | 5.08 | 5.46 | 1.24 | 1.07 |
| pH value of 300 mM sodium di-hydrogen phosphate buffer | | | | |
| 3.0 | 2.98 | 3.52 | 3.40 | 1.18 |
| 3.5 | 3.32 | 3.84 | 3.05 | 1.16 |
| 4.0 | 3.57 | 4.09 | 2.29 | 1.15 |
| 5.0 | 4.74 | 5.26 | 1.46 | 1.11 |

$k'_1$: retention factor of (*R*)-enantiomer; $k'_2$: retention factor of safinamide mesilate; Rs: resolution; $\alpha$: selectivity.

**Table 2**
The selectivity of safinamide mesilate enantiomers on Chiralpak AD-RH column with different pH values of burrer salt solutions in modifiers [(sodium di-hydrogen phosphate buffer:methanol:acetonitrile (65:25:10, v/v/v)] at a flow rate of 0.5 mL/min at 25 °C.

| Burrer salt solution | $k'_1$ | $k'_2$ | Rs | $\alpha$ |
|---|---|---|---|---|
| pH value of 100 mM sodium di-hydrogen phosphate buffer | | | | |
| 3.0 | 2.94 | 3.27 | 1.65 | 1.11 |
| 3.5 | 3.34 | 3.64 | 1.43 | 1.09 |
| 4.0 | 3.86 | 4.15 | 1.22 | 1.08 |
| 5.0 | 5.11 | 5.40 | 0.71 | 1.06 |
| pH value of 200 mM sodium di-hydrogen phosphate buffer | | | | |
| 3.0 | 2.70 | 3.11 | 1.87 | 1.15 |
| 3.5 | 3.07 | 3.43 | 1.74 | 1.12 |
| 4.0 | 3.65 | 4.04 | 1.59 | 1.11 |
| 5.0 | 4.84 | 5.18 | 1.03 | 1.07 |
| pH value of 300 mM sodium di-hydrogen phosphate buffer | | | | |
| 3.0 | 2.38 | 2.82 | 2.08 | 1.18 |
| 3.5 | 2.73 | 3.15 | 1.85 | 1.15 |
| 4.0 | 3.35 | 3.78 | 1.60 | 1.13 |
| 5.0 | 4.42 | 4.87 | 1.12 | 1.10 |

$k'_1$: retention factor of (*R*)-enantiomer; $k'_2$: retention factor of safinamide mesilate; Rs: resolution; $\alpha$: selectivity.

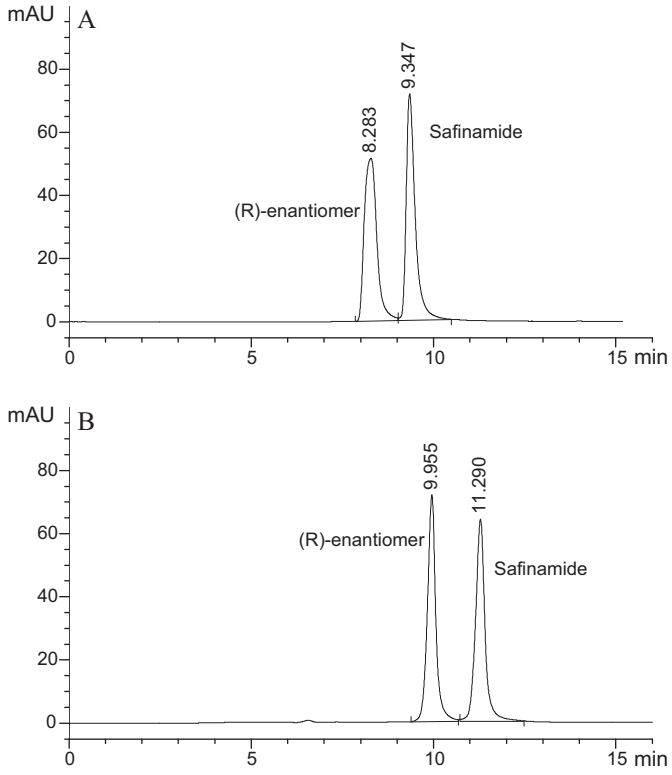


**Fig. 2.** Enantiomeric resolution of racemic safinamide mesilate on (A) Chiralpak AD-RH column and (B) Chiralcel OD-RH column. Mobile phase consisted 300 mM sodium di-hydrogen phosphate buffer (pH 3.0):methanol:acetonitrile (65:25:10, v/v/v); flow rate, 0.5 mL/min; UV, 224 nm; Column temperature, 25 °C.

11.29 min, respectively. Baseline separation of safinamide and (*R*)-enantiomer was obtained with a total run time of 15 min.

The chromatographic separation of safinamide mesilate enantiomers in the optimized conditions using UV detector on Chiralcel OD-RH and Chiralpak AD-RH columns was shown in Fig. 2. The system suitability test results of the chiral HPLC method on Chiralcel OD-RH and Chiralpak AD-RH columns were presented in Table 3.

### 3.2. Validation results of the method

The HPLC condition of the final method was evaluated for its precision, LOD, LOQ, linearity, recovery, and robustness.

The repeatability and intermediate precision were expressed as relative standard deviation (R.S.D.). For this study, safinamide mesilate (50 μg/mL) spiked with (*R*)-enantiomer (0.6%, 300 ng/mL) was analyzed in six replicates each day over a period of three consecutive days to establish repeatability. The R.S.D. values were less than 0.5% for the retention times of both the enantiomers, 1.0% for safinamide peak area and 5.0% for (*R*)-enantiomer peak area (Table 4). In the intermediate precision study, results showed that R.S.D. values were in the same order of magnitude as those obtained for repeatability studies (Table 4). All these values indicated that the method was precise.

**Table 3**
System-suitability test results using Chiracel OD-RH and Chiralpak AD-RH columns with sodium di-hydrogen phosphate buffer (pH 3.0):methanol:acetonitrile (65:25:10, v/v/v) as mobile phase at a flow rate of 0.5 mL/min at 25 °C.

| Column name | Compound ($n = 3$) | $k'$ | Rs | $N$ | $T$ | $\alpha$ |
|---|---|---|---|---|---|---|
| Chiralcel OD-RH | (*R*)-enantiomer | 2.98 | | 11,258 | 1.02 | |
| | Safinamide mesilate | 3.52 | 3.40 | 11,303 | 1.01 | 1.18 |
| Chiralcel AD-RH | (*R*)-enantiomer | 2.38 | | 2868 | 0.97 | |
| | Safinamide mesilate | 2.82 | 2.08 | 8559 | 0.55 | 1.18 |

$n = 3$ determinations; $k'$: retention factor; Rs: USP resolution; $N$: number of theoretical plates (USP tangent method); $T$: USP tailing factor; $\alpha$: enantioselectivity.

**Table 4**
Validation results of the developed chiral LC method.

| Validation parameter | Results |
|---|---|
| Repeatability ($n = 6$, % R.S.D.) | |
| Retention time (*R*-enantiomer) | 0.4 |
| Retention time (safinamide) | 0.3 |
| Area (*R*-enantiomer) | 4.2 |
| Area (safinamide) | 0.8 |
| Repeatability ($n = 18$, % R.S.D.) | |
| Retention time (*R*-enantiomer) | 0.7 |
| Retention time (*S*-enantiomer) | 0.5 |
| Area (*R*-enantiomer) | 4.9 |
| Area (*S*-enantiomer) | 1.0 |
| LOD–LOQ (*R*-enantiomer) | |
| Limit of detection (ng/mL) | 15 |
| Limit of quantification (ng/mL) | 50 |
| Precision at LOQ (% R.S.D.) | 5.7 |
| Linearity (*R*-enantiomer) | |
| Calibration range (ng/mL) | 50–600 |
| Calibration points | 6 |
| Correlation coefficient | 0.9997 |
| Slope (% R.S.D.) | 3.4 |
| Intercept (% R.S.D.) | 9.4 |

The LOD and LOQ concentrations were estimated to be 15 and 50 ng/mL for (*R*)-enantiomer, respectively, when signal-to-noise (S/Ns) ratios of 3 and 10 were used as the criteria (Table 4). The method precision for (*R*)-enantiomer at LOQ was less than 6.0% R.S.D. Therefore, this method had adequate sensitivity for the detection and estimation of (*R*)-enantiomer in safinamide mesilate.

Good linearity of (*R*)-enantiomer was evaluated over six levels of (*R*)-enantiomer solutions from 50 to 600 ng/mL, with the linear regression equation $y = 0.1518x - 0.5730$ (correlation coefficient $r^2 = 0.9997$), where $x$ is the concentration of the undesired enantiomer in ng/mL, and $y$ is the corresponding peak area of the undesired enantiomer in mV/s. Linearity was checked for (*R*)-enantiomer over the same concentration range for three consecutive days. The R.S.D. of the slope and *Y*-intercept of the calibration curve were 3.4% and 9.4% (Table 4), respectively.

The standard addition and recovery experiments were conducted for (*R*)-enantiomer in bulk samples in triplicate at 0.4% (200 ng/mL), 0.6% (300 ng/mL) and 0.8% (400 ng/mL) of analyte concentration. A HPLC chromatogram of safinamide mesilate bulk drug sample (50 μg/mL) spiked with (*R*)-enantiomer (0.6%) was shown in Fig. 3. The recovery was calculated from the slope and *Y*-intercept of the calibration curve obtained linearity study and percentage recovery was ranged from 94.2% to 103.7% (Table 5). The method was proved to be accurate in estimating the amount of

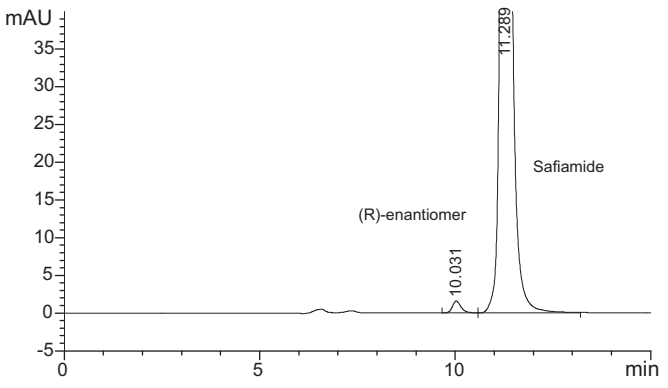


**Fig. 3.** Typical HPLC chromatogram of safinamide mesilate bulk sample (50 μg/mL) spiked with (*R*)-enantiomer (0.6%).

**Table 5**
Recovery results of (*R*)-enantiomer in bulk drugs.

| Added (ng/mL) | Recovered (ng/mL) | Recovery (%) | R.S.D. (%) |
|---|---|---|---|
| 200.0 | 188.4 | 94.2 | 4.3 |
| 300.0 | 311.2 | 103.7 | 2.6 |
| 400.0 | 405.6 | 101.4 | 2.4 |

*n* = 3 determinations.

**Table 6**
Robustness of the chiral LC method.

| Parameter | Resolution between safinamide and (*R*)-enantiomer |
|---|---|
| Flow rate (mL/min) | |
| 0.45 | 3.65 |
| 0.50 | 3.40 |
| 0.55 | 3.22 |
| Column temperature (°C) | |
| 20 | 3.58 |
| 25 | 3.40 |
| 30 | 3.24 |
| Sodium di-hydrogen phosphate buffer (pH 3.0):methanol:acetonitrile (v/v/v) | |
| 65:26:9 | 3.62 |
| 65:25:10 | 3.40 |
| 65:24:11 | 3.17 |
| pH value of phosphate buffer solution | |
| 2.9 | 3.57 |
| 3.0 | 3.40 |
| 3.1 | 3.33 |

the undesired enantiomer of safinamide mesilate between 50 and 600 ng/mL.

The method robustness studies were demonstrated by adjusting flow rate, column temperature and mobile phase composition variations. The chromatographic resolution of safinamide and (*R*)-enantiomer peaks was used to evaluate the method robustness. The resolution between safinamide and (*R*)-enantiomer was more than 3.0 under all tested separation conditions (Table 6), demonstrating sufficient robustness.

The stability of the solutions and mobile phase used in this method was tested over a long time. No significant change in (*R*)-enantiomer content was observed in safinamide mesilate sample during solution stability and mobile phase stability experiments. Hence, safinamide mesilate sample solution and mobile phase were stable at least for 48 h.

## 4. Conclusion

A simple, rapid, and accurate enantioselective reversed-phase HPLC method was successfully developed, which was capable of separating the undesired enantiomer from safinamide mesilate. Two different polysaccharide-based chiral columns Chiralcel OD-RH and Chiralpak AD-RH columns were found to be selective for the enantiomers of safinamide mesilate. Method validation was carried out using Chiralcel OD-RH column due to the better chromatographic results achieved on the column. The developed and validated method not only can be used for the chiral purity testing of safinamide mesilate, but also can be used for the quantitative determination of chiral impurity in bulk materials.

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