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# Chiral HPLC Method Development and Validation for Content Of S-Isomer in Fezolinetant on Cellulose Based Stationary Phase

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## Abstract

*Fezolinetant (RFZL) is a small molecule, selective neurokinin-3 (NK3) receptor antagonist recommended for the management of menopausal vasomotor symptoms (hot flashes). In the proposed research work, a normal-phase HPLC method was developed for content of Fezolinetant (S) isomer (SFZL) in Fezolinetant (R) isomer (RFZL) and validated in accordance with ICH Q2(R1) guidelines. Satisfactory resolution of the enantiomer was achieved using a Chiralpak IB chiral stationary phase. The mobile phase consisted of a 70:30 (v/v) mixture of n-hexane and ethanol 70:30 (v/v), operated in isocratic mode at a flow rate of 1.0 mL/min. The column temperature was maintained at 45°C, and the total analysis run time was 15 minutes. The developed method was fully validated and demonstrated to be suitable for the quantitative determination of the Fezolinetant (S) isomer (SFZL) in Fezolinetant (R) isomer (RFZL). Linearity for Fezolinetant (S) isomer (SFZL) was plotted with concentration between 0.67 µg/ml to 13.3 µg/ml. Limit of quantification and limit of detection of Fezolinetant (S) isomer (SFZL) were determined 0.67 µg/ml and 0.22 µg/ml respectively and average recovery of Fezolinetant (S) isomer (SFZL) in Fezolinetant (R) isomer (RFZL) was 100% ± 4%. Newly developed normal phase HPLC method was successfully adopted to control Fezolinetant (S) isomer (SFZL) in Fezolinetant (R) isomer (RFZL). by HPLC.*

***Keywords:** Antagonist; Chiralpak IB; Fezolinetant (R) isomer (RFZL); Fezolinetant (S) isomer (SFZL) and ICH Q2(R1) guidelines.*

## 1. Introduction

Fezolinetant (RFZL) is an oral medicine used for the cure severe vasomotor systems and hot flashes in women due to menopause. Mainly it works on binding and blocking activities of the neurokinin-3 receptor antagonist, which is involved in the neurophysiological regulation of thermoregulatory homeostasis within the central nervous system (U.S. Food and Drug Administration, 2023; Therapeutic Goods Administration, 2024; Health-Central LLC, 2025; Wishart Research Group, 2025). RFZL was first developed by Astellas Pharma (AdisInsight, 2026), it was approved to medicinal use in USA in 2023 May (U.S. Food and Drug Administration, 2023) and in Europe 2023 December (European Medicines Agency, 2024). Chemical name of RFZL is (4-Fluorophenyl)-[(8R)-8-methyl-3-(3-methyl-1,2,4-thiadiazol-5 yl)-5,6- dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]methanone with a molecular formula of $C_{16}H_{15}FN_6OS$ (U.S. Food and Drug Administration, 2023; ChemicalBook). By reviewing structure and chemical name, IUPAC name, chiral isomer is expedited at the (8R) position in the triazolopyrazine moiety (ChemicalBook; MedKoo Biosciences). RFZL in pure form was indented used in the clinical development (Depypere, H., et al.,2019). United States patent numbers 8871761B2 (expiry 04 April 2031), US9422299B2 (expiry 28 March 2034) and US10787458B2 (expiry 25 September 2035) described the process

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for the preparation of Fezolinetant (Astellas Pharma Inc, 2014; Astellas Pharma Inc, 2016; Ogeda SA, 2020). Specific, detailed HPLC conditions for the RFZL drug are provided in proprietary patents US9422299B2 and US10787458B2. The enantiomers of chiral drugs can exhibit different pharmacodynamic, pharmacokinetic, and safety profiles. For instance, isomers may differ in receptor binding affinity, metabolic stability, or toxicity. International Conference on Harmonization (ICH) guideline Q6A (International Council for Harmonisation, 1999) mandate rigorous characterization and quantification of chiral impurities, because even small amounts of the "wrong" enantiomer may impact efficacy or lead to adverse effects. As RFZL enters broader clinical use, robust analytical methods for the resolution, quantification, and monitoring of chiral isomer content is becomes essential. High-performance liquid chromatography (HPLC) using chiral stationary phases is a widely accepted approach for chiral impurity profiling. In proposed work we developed and fully validated analytical method for quantification of SFZL in RFZL by chiral HPLC. Hence, developed and validated analytical method would be used in research and commercial samples.As shown in Figure 1 Chemical structure of RFZL, SFZL and its process related impurities for specificity study (IMP.1, IMP.2, IMP.3 and IMP.4).

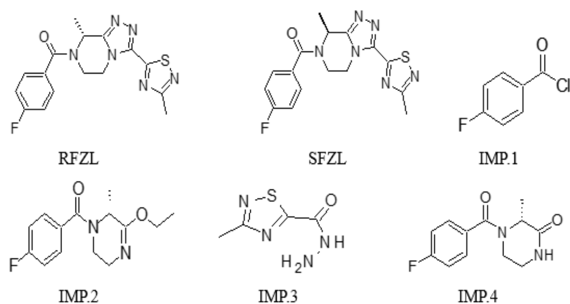


**Figure 1 Chemical structure of RFZL, SFZL and its process related impurities for specificity study (IMP.1, IMP.2, IMP.3 and IMP.4)**

High-Performance Liquid Chromatography (HPLC) is most important analytical tool broadly used in pharmaceutical industry for identification, separation and quantification of enantiomeric impurities from drug molecule. In normal phase (chiral) HPLC mobile phase is nonpolar while stationary phases are polar. Separation between enantiomers and drug molecule was achieved by using specific chiral stationary phases, which improves selective interactions between drug molecule and its enantiomers, resulted in good resolution and sharp peak shape. Chiral stationary phases (CSPs) attain separation in isomers through stereospecific interactions such as π–π interactions, hydrogen bonding, dipole–dipole interactions, and steric complementarity. Now several types of CSPs were developed including polysaccharide-based, cyclodextrin-based, protein-based, macrocyclic antibiotic-based, and Pirkle-type (brush-type) phases. Most commonly used phases are polysaccharide-derived phases, such as amylose and cellulose carbamate derivatives, those are popularly used due to their wide enantioselectivity, high efficiency, and compatibility with normal-phase, reversed-phase, and polar-organic modes (Yashima, E., 2001; Grybinik, S. et al, 2021; Ikai, T. et al, 2006). In proposed research work Chiralpak IB column was used from Diacel manufacturer with cellulose tris (3,5-dimethylphenylcarbamate stationary phase (Daicel Corporation, 2015). Fezolinetant exhibits stereoisomerism, possessing a single chiral centre with an R-configuration. RFZL have one stereogenic centre and hence R and S configuration are possible. Therefore, control of SFZL is required in RFZL (European Medicines Agency, 2023). In proprietary patents US9422299B2 and US10787458B2 HPLC methods are available, diethylamine (DEA) was used in preparation of mobile phase. Basic mobile phase additives, such as DEA are widely utilized in conjunction with polysaccharide-based chiral stationary phases to enhance peak symmetry and improve enantiomeric

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resolution. However, DEA has been reported to exhibit a "memory effect," wherein residual amine remains adsorbed on the stationary phase, thereby influencing subsequent chromatographic separations (Stringham, R. W., et al, 2004). Hence, it is decided that to develop new HPLC method with removal of basic additives in mobile phase with short run time and higher resolution between stereoisomers. Based on the literature and current knowledge, except patent methods, no validated HPLC method has been reported for the detection and quantification of SFZL in RFZL drug-product matrices that is suitable for routine quality-control and stability testing. Therefore, the objective of the present study was to develop and validate a stability-indicating normal phase liquid chromatographic method that is specific, robust, accurate, precise, and linear for the selective detection and quantification of the SFZL impurity in RFZL drug products. Also, developed method was validated as per ICH Q2 R1 guidelines (International Conference on Harmonisation, 2005). As shown in Table 1 Chemical details of RFZL, SFZL and its process related impurities for specificity study (IMP.1, IMP.2, IMP.3 and IMP.4)

**Table 1 Chemical details of RFZL, SFZL and its process related impurities for specificity study (IMP.1, IMP.2, IMP.3 and IMP.4)**

| Name of impurity | Molecular formula | Molecular weight (g/mol) | Chemical names |
|---|---|---|---|
| RFZL | $C_{16}H_{15}FN_6OS$ | 358.39 | (4-Fluorophenyl)[(8R)-8-methyl-3-(3-methyl-1,2,4-thiadiazol-5-yl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]methanone |
| SFZL | $C_{16}H_{15}FN_6OS$ | 358.39 | (4-Fluorophenyl)[(8S)-8-methyl-3-(3-methyl-1,2,4-thiadiazol-5-yl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]methanone |
| IMP.1 | $C_7H_4FClO$ | 158.55 | 4-Fluorobenzoyl chloride |
| IMP.2 | $C_{14}H_{17}FN_2O_2$ | 264.29 | (6R)-5-ethoxy-1-(4-fluorobenzoyl)-6-methyl-1,2,3,6-tetrahydropyrazine |
| IMP.3 | $C_4H_6N_4SO$ | 158.18 | 3-Methyl-1,2,4-thiadiazole-5-carbohydrazide |
| IMP.4 | $C_{12}H_{13}FN_2O_2$ | 236.22 | (3R)-4-(4-fluorobenzoyl)-3-methylpiperazin-2-one |

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## 2. Material And Methods

### 2.1. Reagents, chemicals and standards

In market easily available and cost-efficient chemicals were used in development of method and validation of proposed research work. The HPLC grade n-Hexane and ethanol were procured from Merck chemicals ltd, Mumbai, India. The drug molecule RFZL, and impurity standard of SFZL and its process related impurities (IMP.1, IMP.2, IMP.3 and IMP.4) were synthesised and characterised at research and development department of Megafine pharma (P) Ltd. Nashik, India.

### 2.2. HPLC instrument

Agilent (1200 series, Agilent technologies, Germany) HPLC instrument equipped with autosampler, quaternary pump, column over thermostat, Ultraviolet (UV) and Variable wavelength detector (VWD) and detection is monitored on Chromeleon 7.3 software (Thermo scientific USA).

### 2.3. Chromatographic conditions

Chiral stationary phase was selected for method development and validation was Chiralpak IB, 5µ, 250mm, 4.6mm (Diacel Chiral Technologies). Mobile phase was prepared by composition of n hexane and ethanol 70:30, v/v, ethanol was used as diluent, flow rate was chosen 1.0 mL/min, desired separation was achieved at column over temperature 45°C, autosampler temperature was set at 10°C, injection volume was 10µL, detection wavelength was 284 nm and isocratic mode of elution was used for analysis with run time 15 minutes.

### 2.4. Analytical solution preparation

SFZL standard stock solution was prepared in diluent with 150 µg/mL concentration and it was further diluted to sufficient quantity required for further dilution to desired final standard concentration was 3 µg/mL (refer figure 4). Resolution mixture solution was prepared by taking 2000 ppm RFZL reference standard by spiking 3 µg/mL of SFZL standard (refer figure 3). To determine content of SFZL in RFZL, test sample was prepared in diluent with 2000 µg/mL concentration (refer figure 5). Acceptance limit for SFZL in test sample of RFZL was not more than 0.15% as per ICH guidelines ICH Q3 (International Conference on Harmonisation, 2006).. For chromatogram of blank refer As shown in Figure 2 Typical HPLC chromatogram of blank, Figure 3 Typical HPLC chromatogram of resolution mixture solution.

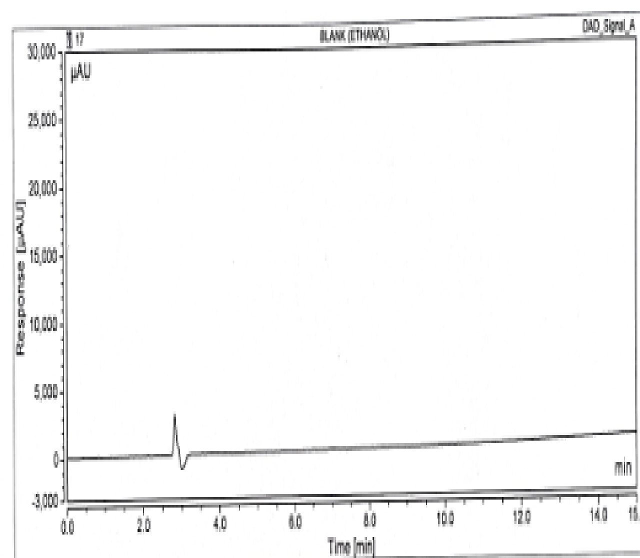


**Figure 2** Typical HPLC chromatogram of blank

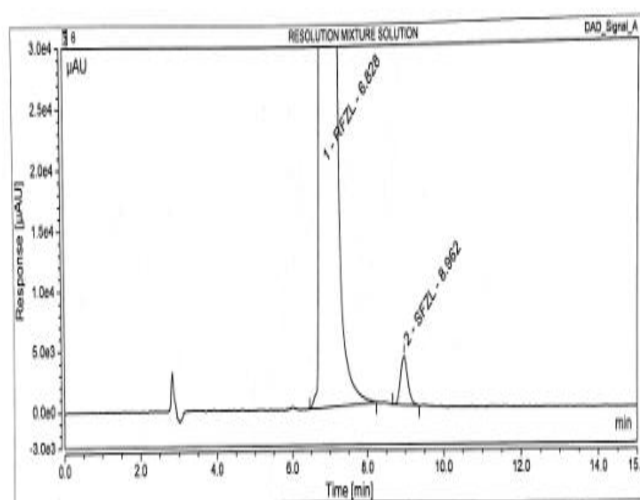


**Figure 3** Typical HPLC chromatogram of resolution mixture solution

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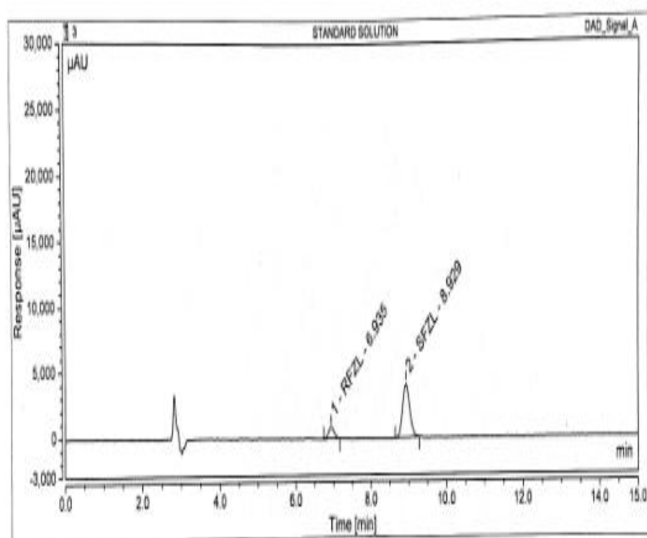


**Figure 4** Typical HPLC chromatogram of standard solution

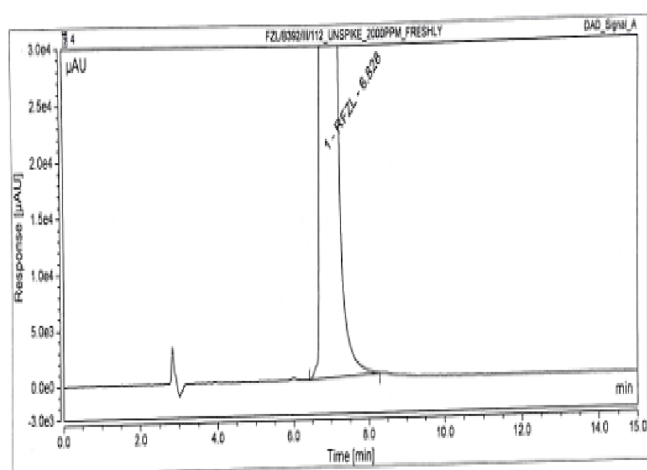


**Figure 5** Typical HPLC chromatogram of test solution

### 2.5.Injection sequence

For analysis of test sample, injection sequence was prepared by two consecutive injections of blank (diluent), single injection of resolution mixture solution to ensure system suitability, six replicate injections of standard solution and one injection of test sample. Standard solution injected after sample injections as bracketing standard to ensure performance of instrument. As system suitability parameter, in resolution mixture solution, resolution between RFZL and SFZL was not less than 3.0 and % RSD for SFZL peak in six replicate injections of standard solution was not more than 5.0%.

## 3. Results And Discussion

### 3.1.Method development

### 3.2. Selection of wavelength

The method development for the quantification of SFZL in RFZL was initiated by recording its ultraviolet (UV) absorption spectrum. A 10 ppm SFZL standard solution was prepared in methanol and a UV scan was performed in the range of 200–400 nm. The analyte exhibited a maximum absorbance at 284 nm, 237 nm and 202 nm (refer figure 6), to reduce baseline noise and blank interference at retention time of analyte peak, 284 nm wavelength was selected for subsequent method development and validation study (Snyder, L. R., et al., 2009; Moldoveanu, S. C., et al., 2025).

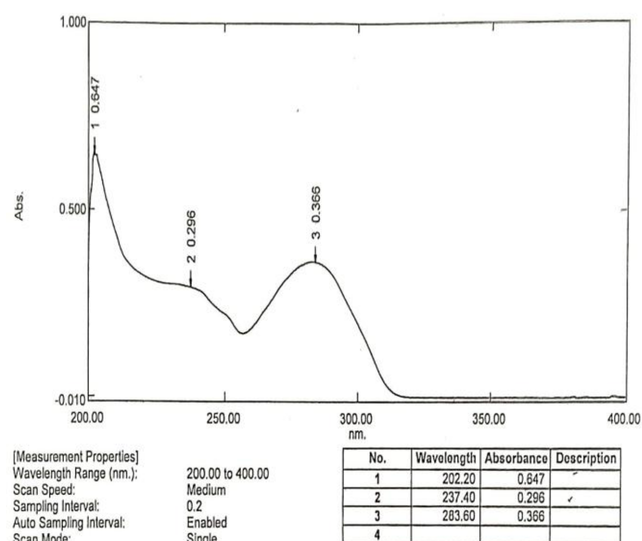


**Figure 6** Representative UV spectrum of SFZL

Before start method development Enantiomeric Ratio (er) and Enantiomeric Excess (ee) of RFZL test sample was determined and it was noted as er = 100:0 and ee = 100% (Eliel, E. L., et al, 1994).

#### 3.2.1. Selection of stationary phase

The main moto of the proposed research was to achieve effective separation and quantification of

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isomer in drug molecule. Various commercially available reagents and chiral stationary phases, including Chiralpak IB, Chiralpak IC, Chiralpak IE, and Chiralpak IA, Chiralpak AD-H and Chiralpak OD-H were evaluated for method development (Ahuja, S. 2007; Ahuja, S. 2010; Jadhav, R. A., et al., 2022; Landge, S. B., et al., 2020; Ahirrao, V., et al., 2021; Subramanian, G., 2008; Kozlov, O., et al., 2020; Ugur, M., et al., 2024; Srinivasu, M. K., et al., 2005; Pathare, D. B., et al., 2006; Dossou, K. S. S., et al., 2011).

- When the mixed standard solution (3 µg/mL each RFZL and SFZL) was injected onto Chiralpak IC (5 µm, 250 × 4.6 mm) and Chiralpak IE (5 µm, 250 × 4.6 mm) columns, both SFZL and RFZL exhibited late elution with broad, distorted peak shapes and reduced resolution[1 – 5].
- Using the Chiralpak IA (5 µm, 250 × 4.6 mm) column, both analytes eluted within 10 minutes with acceptable peak morphology, however, in specificity study peak of impurity IMP.3 eluted too closely to the SFZL peak, compromising resolution.
- While using Chiralpak AD-H column (5 µm, 250 × 4.6 mm), the peaks of RFZL and SFZL were adequately resolved, however, both analytes eluted after 10 minutes and exhibited broad peak shapes[6 – 10].
- By using Chiralpak OD-H column (5 µm, 250 × 4.6 mm) both peaks of RFZL and SFZL eluted within 10 minutes with higher resolution, although the SFZL peak showed increased peak width.
- Subsequently, the Chiralpak IB (5 µm, 250 × 4.6 mm) column, which is widely employed in industry for chiral separations, was evaluated. On this column, SFZL and RFZL eluted within 10 minutes with significantly improved resolution, and all known impurities of RFZL (IMP.1, IMP.2, IMP.3 and IMP.4) were well separated from the SFZL peak. Therefore, the Chiralpak IB column was selected as the optimal stationary phase for the analytical method[11 – 15].

**Table 2 Comparison of resolution, peak width, asymmetry and resolution of the SFZL and RFZL in the mixed standard solution using different columns**

| Sr. No. | Column | Retention time (RT) in min | | Peak width | | Asymmetry | | Resolution between SFZL and RFZL |
|---|---|---|---|---|---|---|---|---|
| | | RFZL | SFZL | RFZL | SFZL | RFZL | SFZL | |
| 1. | Chiralpak IB (5 µm, 250 × 4.6 mm) | 6.86 | 8.78 | 0.289 | 0.356 | 1.33 | 1.25 | 5.94 |
| 2. | Chiralpak IC (5 µm, 250 × 4.6 mm) | 24.81 | 26.87 | 1.127 | 1.209 | 1.08 | 1.11 | 1.76 |
| 3. | Chiralpak IE (5 µm, 250 × 4.6 mm) | 23.66 | 22.25 | 0.844 | 0.783 | 0.94 | 0.89 | 1.73 |
| 4. | Chiralpak IA | 9.72 | 6.98 | 0.346 | 0.251 | 1.12 | 1.12 | 9.18 |

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| | (5 µm, 250 × 4.6 mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 5. | Chiralpak AD-H (5 µm, 250 × 4.6 mm) | 15.64 | 11.09 | 0.675 | 0.473 | 1.27 | 1.25 | 7.93 |
| 6. | Chiralpak OD-H (5 µm, 250 × 4.6 mm) | 6.04 | 9.26 | 0.360 | 0.595 | 1.34 | 1.22 | 6.73 |

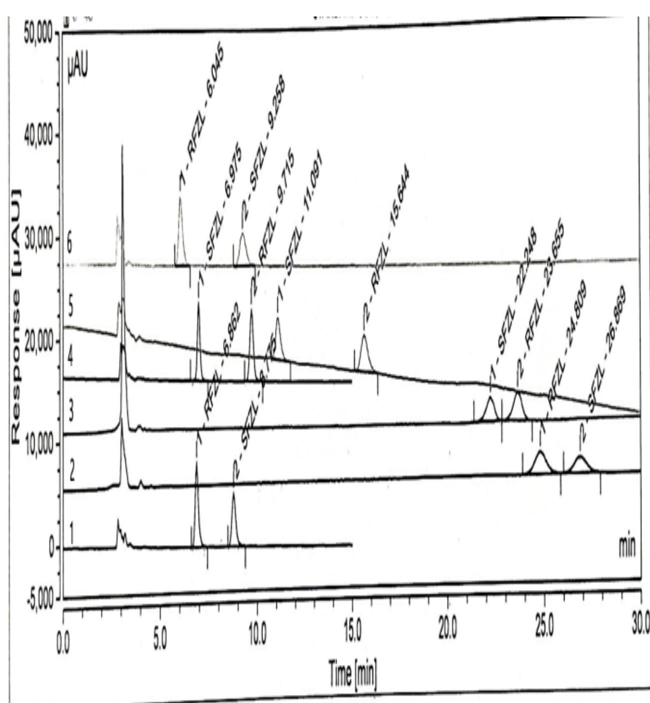


**Figure 7 Typical overlay HPLC chromatogram of the RFZL and SFZL in the mixed standard solution using different columns, 1. Chiralpak IB (5 µm, 250 × 4.6 mm), 2. Chiralpak IC (5 µm, 250 × 4.6 mm), 3. Chiralpak IE (5 µm, 250 × 4.6 mm), 4. Chiralpak IA (5 µm, 250 × 4.6 mm), 5. Chiralpak AD-H (5 µm, 250 × 4.6 mm), 6. Chiralpak OD-H (5 µm, 250 × 4.6 mm) Table 3 Comparison of retention time (min), asymmetry and resolution of RFZL and SFZL on Chiralpak IB column by using different compositions of mobile phase.**

### 3.2.2. Selection of mobile phase

During the preliminary method development for the chromatographic separation of RFZL and SFZL, an initial mobile phase consisting of n-hexane and ethanol (90:10, v/v) was evaluated. However, under these conditions, both analytes exhibited late elution, with retention times exceeding 20 minutes. To achieve earlier elution and improved resolution, the mobile phase composition was systematically optimized by varying the n-hexane-ethanol ratio across multiple experimental runs (refer table 3). Following several iterations, a mobile phase comprising n-hexane and ethanol (70:30, v/v) yielded satisfactory separation, with both RFZL and SFZL eluting within 10 minutes. Using a mobile phase composed of n-heptane and ethanol (70:30, v/v), satisfactory resolution of RFZL and SFZL was achieved[16 – 20], with both analytes eluting within 10 minutes. However, the cost of n-heptane is significantly higher than that of n-hexane. Therefore, to adopt a more cost-effective approach and easily availability, n-hexane was selected as the non-polar component of the mobile phase in place of n-heptane. The RFZL test solution at 2000 µg/mL did not completely dissolve in the optimized mobile phase (n-hexane: ethanol, 70:30, v/v) when used as a diluent. Therefore, ethanol was selected as the diluent to ensure complete solubility of the test sample. As ethanol is volatile, the autosampler temperature was maintained at 10° C to minimize solvent evaporation during sample storage.

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**Table 3** **Comparison of retention time (min), asymmetry and resolution of RFZL and SFZL on Chiralpak IB column by using different compositions of mobile phase**

| Mobile phase (v/v) | Retention time (min) | | Asymmetry of SFZL | Resolution between RFZL and SFZL |
|---|---|---|---|---|
| | RFZL | SFZL | | |
| n hexane: ethanol 90:10 | 22.4 | 30.9 | 1.07 | 6.58 |
| n hexane: ethanol 80:20 | 10.1 | 13.4 | 1.11 | 7.60 |
| n heptane: ethanol 70:30 | 6.7 | 8.6 | 1.09 | 6.22 |
| n hexane: ethanol 70:30 | 6.9 | 8.8 | 1.14 | 6.32 |
| n hexane:2-propanol 90:10 | 11.1 | 13.7 | 1.46 | 2.04 |
| n hexane: ethanol 60:40 | 5.5 | 6.9 | 1.11 | 5.48 |

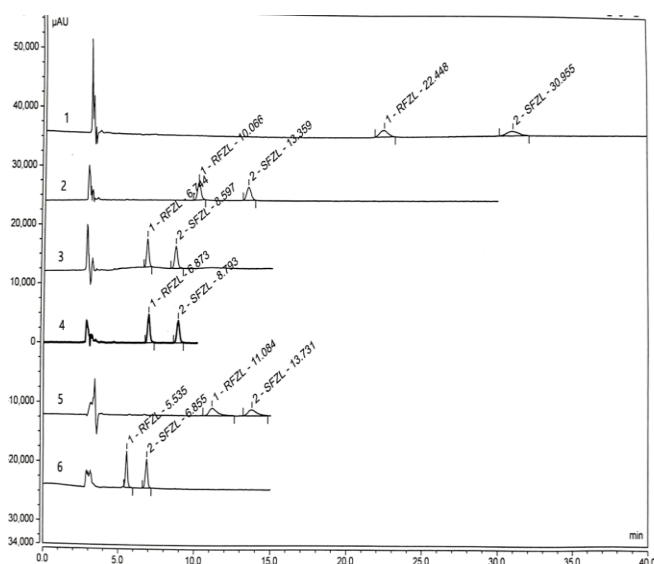


**Figure 8** **Typical overlay HPLC chromatogram of mobile phase composition change study**

1. n hexane: ethanol 90:10 (v/v), 2. n hexane: ethanol 80:20 (v/v), 3. n heptane: ethanol 70:30 (v/v), 4. n hexane: ethanol 70:30 (v/v), 5. n hexane: 2-propanol 90:10 (v/v) and 6. n hexane: ethanol 60:40 (v/v), To evaluate the compatibility and robustness of the developed analytical method, system suitability parameters were assessed during each stage of method development. Optimal resolution and well-defined peak shapes were obtained under the finalized chromatographic conditions using a Chiralpak IB column (250 × 4.6 mm, 5 µm), with a flow rate of 1.0 mL/min, a column temperature of 45 °C, autosampler temperature 10°C and an injection volume of 10 µL. The mobile phase consisted of n-hexane and ethanol (70:30, v/v), while ethanol was used as the

diluent. Under these optimized conditions, the SFZL peak exhibited a sharp profile, and the resolution between RFZL and SFZL was consistently more than 5.0, confirming adequate separation, As shown in Figure 8 Typical overlay HPLC chromatogram of mobile phase composition change study[21 – 30].

### 3.2.3. Column oven temperature study

Column oven temperature is a critical parameter influencing peak asymmetry and chromatographic resolution, and therefore its effect must be systematically evaluated during method development and validation (Ahirrao, V., et al., 2021). A mixed standard solution of RFZL and SFZL (3 µg/mL each) was prepared in the diluent and injected under the optimized chromatographic conditions while varying the column oven temperature from 25 °C to 50 °C. An increase in column temperature resulted in a progressive decrease in retention time and resolution between the two analytes. Optimal peak asymmetry and acceptable resolution were achieved at a column temperature of 45 °C,As shown in Table 4 Effect of column oven temperature on retention time, asymmetry and resolution of RFZL and SFZL by using Chiralpak IB column[31 – 36].

**Table 4 Effect of column oven temperature on retention time, asymmetry and resolution of RFZL and SFZL by using Chiralpak IB column**

| **Column oven temperature (°C)** | **Retention time (min)** | | **Asymmetry of SFZL** | **Resolution between RFZL and SFZL** |
|---|---|---|---|---|
| | RFZL | SFZL | | |
| 25 °C | 9.0 | 13.3 | 1.22 | 7.52 |
| 30 °C | 8.3 | 11.8 | 1.24 | 7.39 |
| 35 °C | 7.8 | 10.6 | 1.19 | 7.18 |
| 40 °C | 7.3 | 9.6 | 1.17 | 6.81 |
| 45 °C | 6.8 | 8.7 | 1.16 | 6.32 |
| 50 °C | 6.5 | 8.0 | 1.13 | 5.79 |

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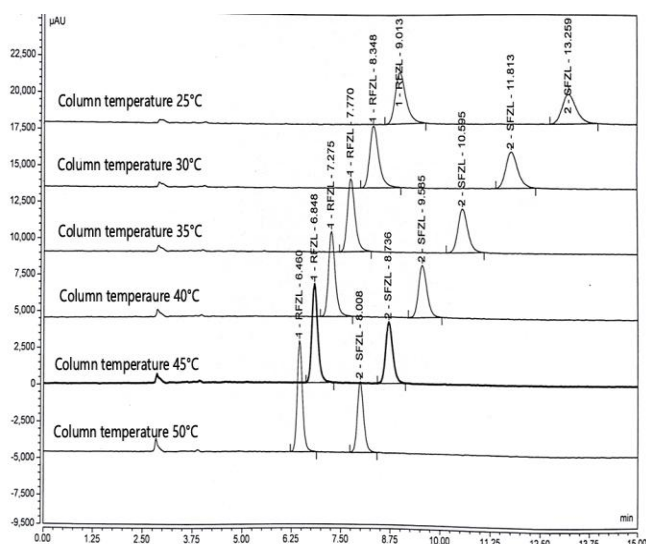


**Figure 9** **Typical overlay chromatogram of column oven temperature study on retention time, Asymmetry and Resolution between RFZL and SFZL**

### 3.2.4. Method validation

As per ICH guideline ICH Q2 (R1), method validation was performed for newly developed HPLC method as shown in Figure 9 Typical overlay chromatogram of column oven temperature study on retention time, Asymmetry and Resolution between RFZL and SFZL,As shown in Table 5 Method validation results data.

**Table 5** **Method validation results data**

| Experiment | Parameter | Results |
|---|---|---|
| System precision (n=6) | RT (min) of SFZL in standard solution | 8.9 |
| | RT (min) of RFZL in RMS | 6.8 |
| | RT (min) of SFZL in RMS | 9.0 |
| | Rs between RFZL and SFZL in RMS | 6.72 |
| | % RSD for SFZL retention time | 0.1 |
| | % RSD for SFZL peak area | 2.2 |
| Method precision (n=6) | % of SFZL (w/w) | 0.13 |
| | % RSD of precision | 0.00 |
| Intermediate precision (n=6) | % of SFZL (w/w) | 0.13 |
| | % RSD of precision | 0.00 |
| | Average % RSD (both precision) | 0.00 |
| Limit of quantification of SFZL | % RSD for precision of peak area | 4.2 |
| | Limit of quantification (µg/mL) | 0.67 |
| | S/N ratio | 36 |
| Limit of detection of SFZL | Limit of detection (µg/mL) | 0.22 |

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| Linearity of SFZL (LOQ to 500%) | Correlation coefficient (r) | | 1.00000 |
|---|---|---|---|
| | Slope | | 20603.60 |
| | Intercept | | -1232.85 |
| Accuracy (n=3) | at LOQ level (%) | | 95.99 |
| | at 50% level (%) | | 100.50 |
| | at 100% level (%) | | 99.00 |
| | at 150% level (%) | | 100.17 |
| Robustness | | | |
| Flow rate (ml/min) | RT (min) of RFZL in RMS | RT (min) of SFZL in RMS | Rs between RFZL and SFZL in RMS |
| 1.1 (+ 0.1 unit) | 6.2 | 8.1 | 6.96 |
| 0.9 (- 0.1 unit) | 7.6 | 9.9 | 6.84 |
| Column temperature (°C) | | | |
| 47 (+ 2 unit) | 6.7 | 8.6 | 6.39 |
| 43 (- 2 unit) | 7.0 | 9.2 | 6.91 |
| Mobile phase ratio (v/v) | | | |
| N hexane: Ethanol 67:33 (+10% ethanol) | 6.3 | 8.2 | 6.80 |
| N hexane: Ethanol 73:27 (-10% ethanol) | 7.5 | 10.0 | 7.79 |

RT: Retention Time, RMS: resolution mixture solution, Rs: Resolution
% LOD and LOQ results were reported with respect to test sample concentration 2000 ppm.

### 3.2.5. Specificity

To establish the specificity of the method, individual solutions of RFZL, SFZL, IMP.1, IMP.2, IMP.3, and IMP.4 (at 20 ppm levels as 1% with respect to test concentration) were injected, followed by analysis of a 2000 µg/mL RFZL test sample spiked with 1% each of SFZL, IMP.1, IMP.2, IMP.3, and IMP.4. No interference was observed at the retention time of the SFZL peak in the blank chromatogram. Additionally, IMP.1, IMP.2, IMP.3, and IMP.4 were well resolved from the SFZL peak, confirming adequate specificity of the developed method (refer figure 10) As shown in Figure 10 Typical specificity chromatogram of RFZL test sample. spiked with IMP.1, IMP.2, IMP.3, and IMP.4

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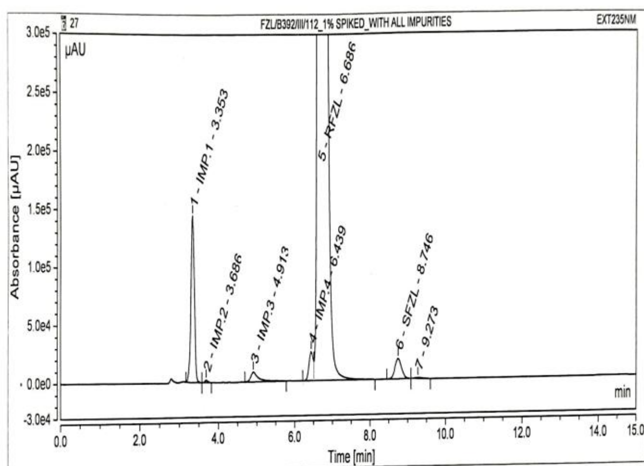

**Figure 10** Typical specificity chromatogram of RFZL test sample. spiked with IMP.1, IMP.2, IMP.3, and IMP.4

### 3.2.6. Precision

As part of the precision study, six replicate injections of the SFZL standard solution (3 µg/mL) were performed, and the %RSD of the peak areas was found to be less than 5.0%. In addition, the RFZL test sample spiked with 0.15% SFZL was prepared and injected six times, and the %RSD for the SFZL was determined to be 0.00%. Intermediate precision was evaluated using a different analyst, chromatographic column, and instrument. Under these conditions, the RFZL test sample spiked with 015% SFZL was again prepared and injected six times, and the %RSD for the SFZL was found to be 0.00%. The average %RSD obtained from method precision and intermediate precision studies was calculated to be 0.00%. For results refer table 5.

### 3.2.7. Linearity

Linearity for SFZL was determined by injecting six different levels corresponding to LOQ, 50%, 100%, 150%, 200%, and 500% of the test sample concentration. A calibration curve was plotted by concentration versus peak response. The correlation coefficient for SFZL was found to be 1.000. For result refer table 5.

### 3.2.8. Limit of Quantification (LOQ) and Limit of Detection (LOD)

Limit of Quantification (LOQ) was established in accordance with ICH Q2(R1) guidelines using commonly applied analytical approaches. Among these, S/N ratio method and visual evaluation is widely employed in routine laboratory practice. For the quantification of SFZL, 25% of the established specification limit (0.67 ppm or 0.0375%) was selected as the LOQ. Precision at the LOQ level was verified by six replicate injections of LOQ level spiked test sample, with % RSD was 4.2% The observed signal-to-noise (S/N) ratio for the SFZL peak at the LOQ concentration was 36. The Limit of Detection (LOD) was defined as 33% of the confirmed LOQ level (0.22 ppm or 0.0125%). Injection of the LOD solution resulted in peak of SFZL was detected.

### 3.2.9. Accuracy/Recovery

RFZL test samples were spiked with SFZL at LOQ, 50%, 100%, and 150% of the specification limit. For each concentration level, three independently prepared spiked test samples were analysed. The mean recovery of all different levels was within the acceptable range of 80.0% to 120.0%. For results refer table 5.

### 3.2.10. Robustness

Robustness of the developed analytical method for the determination of the SFZL in RFZL was assessed by varying key chromatographic parameters within acceptable range. The impact of these variations on the resolution between SFZL in RFZL was systematically evaluated. The optimized column oven temperature for peak separation was 45°C; therefore, robustness was examined by varying the temperature by ±2 °C (43 °C and 47 °C). The mobile-phase flow rate was 1.0 mL/min, and its effect on peak shape and chromatographic resolution was assessed by altering the flow rate by ±0.1 mL/min (0.9 and 1.1 mL/min). The effect of change in compositions in mobile phase was studied systematically, mobile phase composition was n hexane: ethanol 70:30 v/v, the volume of ethanol was changed by ±10% as hexane: ethanol 67:33 v/v and hexane: ethanol 73:27 v/v.

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For each specific change, all remaining chromatographic conditions were kept same. Across all robustness conditions such as column oven temperature, flow rate, and mobile phase composition, resolution between RFZL and SFZL remained more than 5.0, and peak asymmetry for SFZL in was less than 2.0. Based on observed results confirm that the method is robust and ready to use for routine quantification of SFZL content in RFZL drug products. For results refer table 5.

### 3.2.11. Solution stability and mobile phase stability

Solution stability was evaluated by analysing freshly prepared SFZL standard solution, RFZL unspiked test solution, and RFZL test solution spiked with SFZL at different intervals up to 48 hours at a sampler temperature of 10°C. The %RSD of the SFZL peak in the standard solution remained within the acceptable limit (not more than 5.0%). In the unspiked RFZL test solution, SFZL was not detected at any time point, and no change was observed throughout the 48-hour. Similarly, the SFZL content in the spiked RFZL test solution showed no significant variation across all evaluated time intervals as shown in Table 6 Result of solution stability for SFZL content in RFZL test sample.

**Table 6 Result of solution stability for SFZL content in RFZL test sample**

| Time interval from preparation | % of SFZL in unspiked test | % of SFZL in spiked test |
|---|---|---|
| Freshly prepared | Not detected | 0.130 |
| A/6 hours from preparation | Not detected | 0.131 |
| A/18 hours from preparation | Not detected | 0.130 |
| A/24 hours from preparation | Not detected | 0.131 |
| A/30 hours from preparation | Not detected | 0.130 |
| A/36 hours from preparation | Not detected | 0.130 |
| A/48 hours from preparation | Not detected | 0.130 |

These results demonstrate that the SFZL standard solution, the RFZL unspiked test solution, and the RFZL test solution spiked with SFZL are stable for 48 hours at 10 °C autosampler temperature. Furthermore, the retention times of SFZL and RFZL, as well as the measured SFZL content in RFZL, remained consistent throughout the study, confirming that the prepared mobile phase is also stable for up to 48 hours.

### 3.2.12. Application of developed method

By using newly developed method three different test samples of RFZL were analysed for quantification of SFZL in RFZL by HPLC. Results are reported in table 7. One of the test samples was subsequently employed for the recovery (accuracy) study at LOQ, 50%, 100%, and 150% concentration levels. The obtained accuracy results complied with the acceptance criteria specified in the ICH Q2(R1) guideline. Accordingly, the reported batch analysis results are considered accurate and reliable. The limit of detection (LOD) and limit of quantification (LOQ) were established using both visual evaluation and signal-to-noise (S/N) ratio approaches, and were determined to be 0.67 µg/mL and 0.22 µg/mL respectively. The S/N ratio at the LOQ level exceeded 10. To verify

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analytical sensitivity, a RFZL test sample spiked with the SFZL at the LOQ concentration was injected and analysed to confirm the validity of the "not detected" results (Shinde, R. S., et al., 2026), As shown in Table 7 Results of SFZL content in RFZL test.

**Table 7 Results of SFZL content in RFZL test**

| Batch analysis results | samples |
|---|---|
| Sample name | % of SFZL |
| Drug sample.1 | Not detected |
| Drug sample.2 | Not detected |
| Drug sample.3 | Not detected |

## Conclusion

A novel, cost-effective, specific, linear, accurate, and robust HPLC method was developed for the quantification of SFZL in RFZL test sample, with good peak shape and satisfactory resolution. The method was successfully validated as per ICH Q2(R1) guidelines, and all validation parameters complied with the specified acceptance criteria. The validated method is suitable for routine commercial analysis and can be applied for the commercial scale samples for determination of SFZL content in RFZL by using chiral HPLC.

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