A Novel Polar Organic UFLC Method for Chiral Separation and Quantification of Tofisopam Enantiomers in API and Formulations

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ABSTRACT

Objective: *To develop and validate a simple, precise, and efficient chiral Ultra-Fast Liquid Chromatographic (chiral-UFLC) method operating in polar organic mode for the enantiomeric separation of racemic Tofisopam [1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine], an anxiolytic drug.*

Methods: *Chromatographic separation was carried out on a Chiralcel OJ-H column (4-methyl benzoate, 250 mm × 4.6 mm, 5 µm particle size) using a mobile phase consisting of methanol, 2-propanol, and diethylamine in the ratio of 95:5:0.1 (v/v/v). The flow rate was maintained at 0.5 mL/min, and detection was performed at 310 nm. Method validation was conducted following ICH guidelines for accuracy, precision, linearity, and sensitivity.*

Results and Discussion: *Inclusion of 2-propanol in the mobile phase significantly improved chromatographic resolution and efficiency, achieving a resolution greater than 3.0 between the enantiomers. The limits of detection (LOD) and quantification (LOQ) were 0.25 µg/mL and 0.44 µg/mL for the S-enantiomer, and 0.31 µg/mL and 0.48 µg/mL for the R-enantiomer, respectively, based on a 20 µL injection volume. Recovery studies showed 98.00–102.35% for the S-enantiomer and 99.00–101.15% for the R-enantiomer.*

Conclusion: *The developed chiral-UFLC method is simple, reliable, and cost-effective, providing high resolution and reproducibility. It is suitable for routine enantioselective quantification of S- and R-Tofisopam in both active pharmaceutical ingredients and dosage forms.*

Keywords*: Tofisopam, Chiral separation, Polar organic mode, Chiralcel OJ-H column, UFLC and Method validation etc.*

1. INTRODUCTION

Chiral molecules play a vital role in various scientific fields such as toxicology, biology, pharmacology, and medicinal chemistry due to their enantioselective biological activities. The therapeutic efficacy and safety of chiral drugs often depend on their individual enantiomers, which has encouraged the development of single-enantiomer pharmaceuticals. At present, over half of marketed drugs are chiral, and many are administered as racemic mixtures, driving pharmaceutical industries to focus on resolving and developing pure enantiomers [1,2]. Regulatory authorities such as the U.S. Food and Drug Administration (FDA) require comprehensive pharmacological and pharmacokinetic data for each enantiomer in racemic drugs, emphasizing the need for precise and efficient analytical methods for enantiomeric separation [3,4].

Analytical techniques commonly employed for chiral separation include high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and supercritical fluid chromatography (SFC), with HPLC being the most widely adopted. Among HPLC methods, polysaccharide-based chiral stationary phases (CSPs) are particularly effective because of their broad enantioselectivity and flexibility in normal phase, reversed phase, and polar organic modes [5]. The use of organic modifiers such as methanol and acetonitrile enhances solubility, improves resolution, and reduces analysis time [6].

Tofisopam, a 2,3-benzodiazepine derivative chemically known as 1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine, is a racemic anxiolytic drug. Unlike classical benzodiazepines, tofisopam does not bind to the GABA-A receptor and therefore lacks sedative, muscle relaxant, and anticonvulsant effects [7]. The compound possesses a chiral center at the C-5 position, forming two enantiomers: S-(−)-tofisopam (levotofisopam) and R-(+)-tofisopam (dextofisopam). Each enantiomer exists as two conformers (major and minor), resulting in four stereoisomers—(+)-R, (−)-R, (+)-S, and (−)-S [8]. The major conformers [(+)-R and (−)-S] adopt a quasi-equatorial orientation of the 5-ethyl group, while the minor conformers [(−)-R and (+)-S] exhibit a quasi-axial orientation, caused by steric interactions between the 4-methyl and 5-ethyl substituents. These conformers interconvert slowly in solution, enabling chromatographic resolution [9].

Tofisopam is marketed as a racemate under brand names such as Grandaxin®. The R-enantiomer is under clinical investigation for irritable bowel syndrome, while the S-enantiomer demonstrates strong PDE-4 inhibitory and uricosuric activity [10,4]. Previous chromatographic methods using n-hexane-based mobile phases achieved poor resolution and relied on toxic, volatile solvents [11,12]. Therefore, the development of a rapid, eco-friendly, and cost-effective chiral UFLC method operating in polar organic mode is essential. Such an approach using a polysaccharide-based CSP (Chiralcel OJ-H) provides high resolution, improved efficiency, and reduced run time for the enantioselective and conformational analysis of tofisopam in pharmaceutical formulations.

2. MATERIALS AND METHODS

2.1. Reagents and Instruments

The reagents and chemicals used in the present research work are of Analytical Reagent (AR) and Laboratory Reagent (LR) grade, and were used without further purification.

2.1.1. Chemicals and reagents: Racemic tofisopam and its individual enantiomers, in the form of active pharmaceutical ingredients (APIs), were kindly supplied by Symed Labs Ltd. (Sanga Reddy, Hyderabad, Telangana, India). Commercial tofisopam tablets (Tofisopam Tablets IP 50 mg, marketed as Grandaxin®) were purchased from a local pharmacy and manufactured by Intas Pharmaceuticals Ltd. (East Sikkim, India). Analytical-grade solvents, including n-hexane, ethanol, isopropanol, diethylamine, and methanol (HPLC grade), were procured from Sigma-Aldrich Chemicals Pvt. Ltd. (Maharashtra, India). Polyvinylidene fluoride (PVDF) syringe filters with a pore size of 0.22 µm were obtained from HiMEDIA Laboratories Pvt. Ltd. (Mumbai, India) and used for sample filtration prior to chromatographic analysis.

2.1.2. Instruments: Chromatographic analyses were performed using an Ultra-Fast Liquid Chromatography (UFLC) system (Shimadzu, Kyoto, Japan) equipped with a binary pump (model LC-20AD), a UV–Visible detector (model LC-20A), and a manual Rheodyne injector port. The separation was carried out on a Lux Cellulose-OJ-H chiral column (250 mm × 4.6 mm, 5 µm particle size) obtained from Phenomenex (USA). Data acquisition and processing were performed using LabSolutions software (Shimadzu, Japan). For method development and optimization, several polysaccharide-based chiral stationary phase (CSP) columns were evaluated, including Chiralcel OD-H, OZ-H, OJ-H, and OX-H. Among these, two CSPs were further investigated under polar organic mode (POM) conditions to achieve enhanced enantioselectivity and resolution. Sample preparation was facilitated by the use of an ultrasonic bath, ensuring complete dissolution of the analyte in the selected solvent system prior to chromatographic analysis.

2.1.3. Chromatographic Conditions: The chromatographic separation was optimized using a cellulose-based chiral stationary phase (CSP) column, Chiralcel OJ-H (250 mm × 4.6 mm, 5 µm particle size; Daicel, Japan), coated with tris-(4-methylbenzoate) as the chiral selector. The mobile phase consisted of methanol, isopropanol, and diethylamine in the ratio of 95:5:0.1 (v/v/v), operating in the polar organic mode (POM) to achieve efficient enantiomeric resolution.

The flow rate was maintained at 0.5 mL/min, with the column temperature set at 25 °C. Detection was performed at a wavelength of 310 nm, and the injection volume was fixed at 20 µL. Under these optimized conditions, baseline separation of the Tofisopam enantiomers was achieved within 12 minutes, demonstrating high resolution and reproducibility suitable for routine analytical applications.

2.1.4. Standard Preparation: A standard stock solution of racemic Tofisopam was prepared at a concentration of 1 mg/mL using the mobile phase as the diluent. From this stock solution, a series of working standard solutions in the concentration range of 0.5–8 µg/mL were prepared by appropriate serial dilutions. All dilutions were performed in 1.5 mL Eppendorf tubes using calibrated micropipettes to ensure accuracy and precision. The prepared solutions were filtered through 0.22 µm PVDF syringe filters prior to chromatographic analysis to remove any particulate matter.

2.1.5. Sample Preparation: Grandaxin-50, a marketed formulation containing 50 mg of Tofisopam per tablet, was analyzed to determine the percentage assay of its individual enantiomers. A quantity of the finely powdered tablets equivalent to 10 mg of Tofisopam was

accurately weighed and transferred into a 10 mL volumetric flask. The contents were dissolved in the mobile phase, sonicated in an ultrasonic bath for 10 minutes to ensure complete dissolution, and the volume was made up to the mark with the same diluent to obtain a final concentration of 10 µg/mL. The resulting solution was filtered through a 0.22 µm PVDF syringe filter to remove particulate matter prior to injection. The filtered sample was then analyzed using the UFLC system under the optimized polar organic mode conditions for the enantioselective assay of Tofisopam.

2.2. Method development and validation

The developed chiral-UFLC method was validated in accordance with ICH Q2(R1) guidelines for critical analytical parameters, including specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, and system suitability, to ensure the reliability, reproducibility, and suitability of the method for routine analysis of Tofisopam enantiomers [13–15].

2.2.1. System Suitability: The system suitability of the developed chiral-UFLC method was evaluated by injecting a selected concentration of Tofisopam six consecutive times into the UFLC system. The method was considered suitable if it met the following acceptance criteria: the number of theoretical plates (N) exceeded 2000, the tailing factor (T) remained below 2, and the resolution (Rs) between the enantiomeric peaks was greater than 2. These parameters ensured the system's performance, reproducibility, and reliability for accurate enantiomeric analysis.

2.2.2. Linearity: Drug solutions of Tofisopam were initially prepared at a concentration of 100 µg/mL following the standard procedure described previously. These solutions were subsequently diluted to obtain a series of working concentrations in the range of 0.5–8 µg/mL. A calibration curve was constructed by plotting the peak areas of the individual S- and R-enantiomers against their corresponding concentrations. Linear regression analysis was performed, and the coefficient of determination (r^2) was calculated to assess the linearity and suitability of the method for quantitative enantiomeric analysis.

2.2.3. Precision and recovery: Method precision was evaluated by determining the percentage relative standard deviation (%RSD) of the peak areas from six independent sample preparations at three different concentration levels. Precision was assessed for both intra-day (within a single day) and inter-day (over multiple days) analyses. System precision was assessed by calculating the %RSD of replicate injections of the same concentration levels under identical chromatographic conditions. Accuracy of the method was determined through recovery studies, wherein known amounts of each individual S- and R-Tofisopam enantiomer were spiked into the sample matrix, and the percentage recoveries were calculated to evaluate the closeness of the measured values to the true values. The results confirmed the method’s reliability and reproducibility for quantitative enantiomeric analysis.

2.2.4. Limit of detection and limit of quantification: The limit of detection (LOD) was defined as the lowest concentration of Tofisopam at which the analyte peak could be reliably detected, corresponding to a signal-to-noise (S/N) ratio of 3. The limit of quantification (LOQ) was defined as the lowest concentration at which the analyte peak could be quantitatively measured, corresponding to an S/N ratio of 10. Both LOD and LOQ were estimated using the standard

deviation of the residuals, calculated by subtracting the observed peak area from the theoretical peak area, and the slope of the calibration curve obtained from the linearity study. These values confirmed the method's sensitivity for detecting and quantifying both S- and R-Tofisopam enantiomers at low concentrations.

2.2.5. Specificity: Specificity of the developed chiral-UFLC method was evaluated by separately injecting the blank solution and the Tofisopam sample solution into the UFLC system. The method was considered specific if no interfering peaks were observed at the retention times of the S- and R-enantiomers, confirming that the analyte peaks were free from interference from the solvents, excipients, or other components in the sample matrix.

2.2.6. Robustness: Robustness of the developed chiral-UFLC method was assessed by introducing deliberate small variations in chromatographic parameters to evaluate the method's reliability under slightly altered conditions. The parameters tested included flow rate, detection wavelength, and injection volume, each varied at two levels slightly above and below the optimized settings. Specifically, the flow rate was adjusted by ±0.1 mL/min, the detection wavelength by ±1 nm, and the injection volume by ±0.1 µL. The method was considered robust if these changes did not significantly affect the retention times, resolution, or peak areas of the S- and R-Tofisopam enantiomers.

3. RESULTS AND DISCUSSION

3.1. Method Optimization

The developed chiral-UFLC method successfully achieved baseline separation and precise quantification of the (S)- and (R)-enantiomers of tofisopam. Various polysaccharide-based chiral columns (Chiralcel OD-H, OZ-H, OJ-H, and OX-H) and mobile phase combinations were evaluated. Chiralcel OD-H and OX-H showed poor enantioresolution, while the OJ-H column exhibited promising separation. Initial trials with acetonitrile/methanol/diethylamine (85:15:0.1, v/v/v) and methanol/ethanol/triethylamine (85:15:0.1, v/v/v) produced broad peaks. Replacing ethanol with isopropanol significantly improved peak shapes and resolution. Optimal separation was obtained with methanol/isopropanol/diethylamine (95:5:0.1, v/v/v), achieving resolution values >3.

Elution order of enantiomers depended on the mobile phase composition, reflecting transient diastereomeric interactions, including hydrogen bonding, dipole–dipole, and π–π interactions with the cellulose-based stationary phase. The Chiralcel OJ-H column allowed complete baseline separation of all four stereoisomers, with minor R- and S-enantiomers eluting at ~5.4 and 6.5 min, and major R- and S-enantiomers at ~8.6 and 7.5 min, respectively. System suitability results confirmed high efficiency ($N > 5500$), low tailing ($T < 1.22$), and resolution >3 (Table 2, Figure 2).

These findings demonstrate that the Chiralcel OJ-H column with a polar organic mobile phase provides a robust, reproducible, and selective method for enantioselective analysis of tofisopam, enabling accurate quantification of its major enantiomers in pharmaceutical formulations.

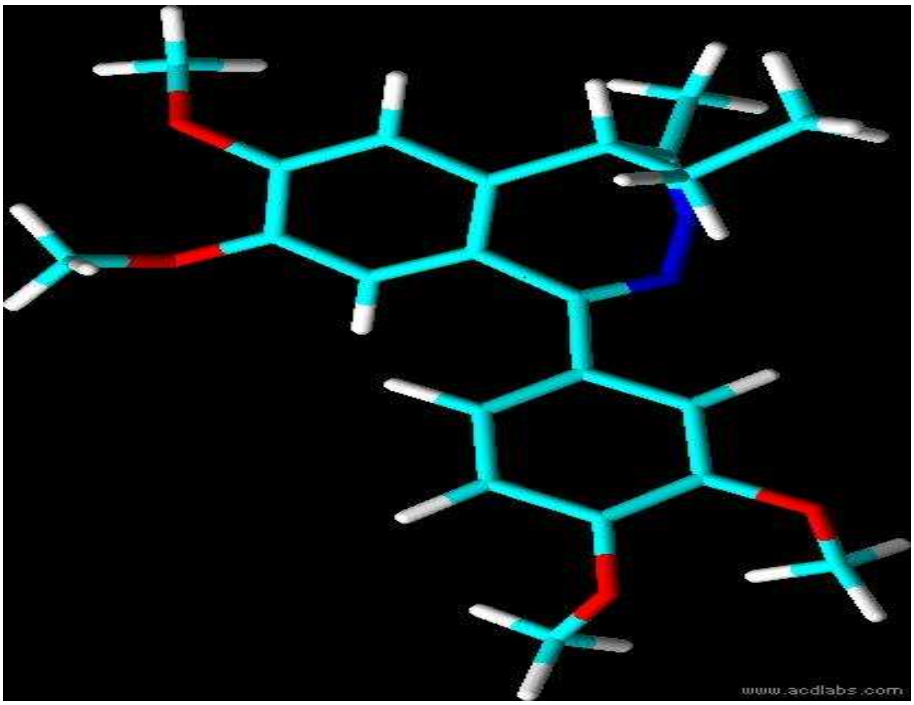

Fig-1: Sticks Model of Tofisopam

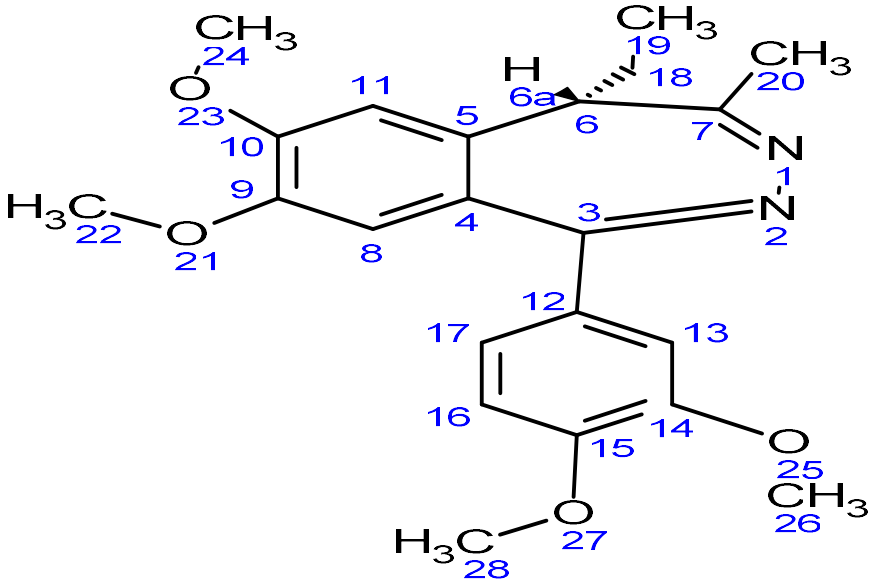

Fig-2: **Racemic form of Tofisopam**

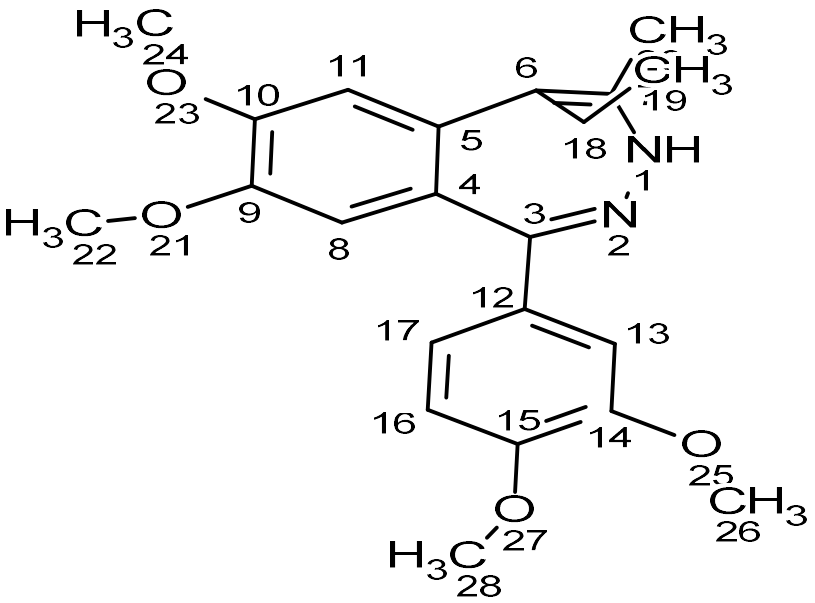

Fig-3: Tautomeric form of Tofisopam

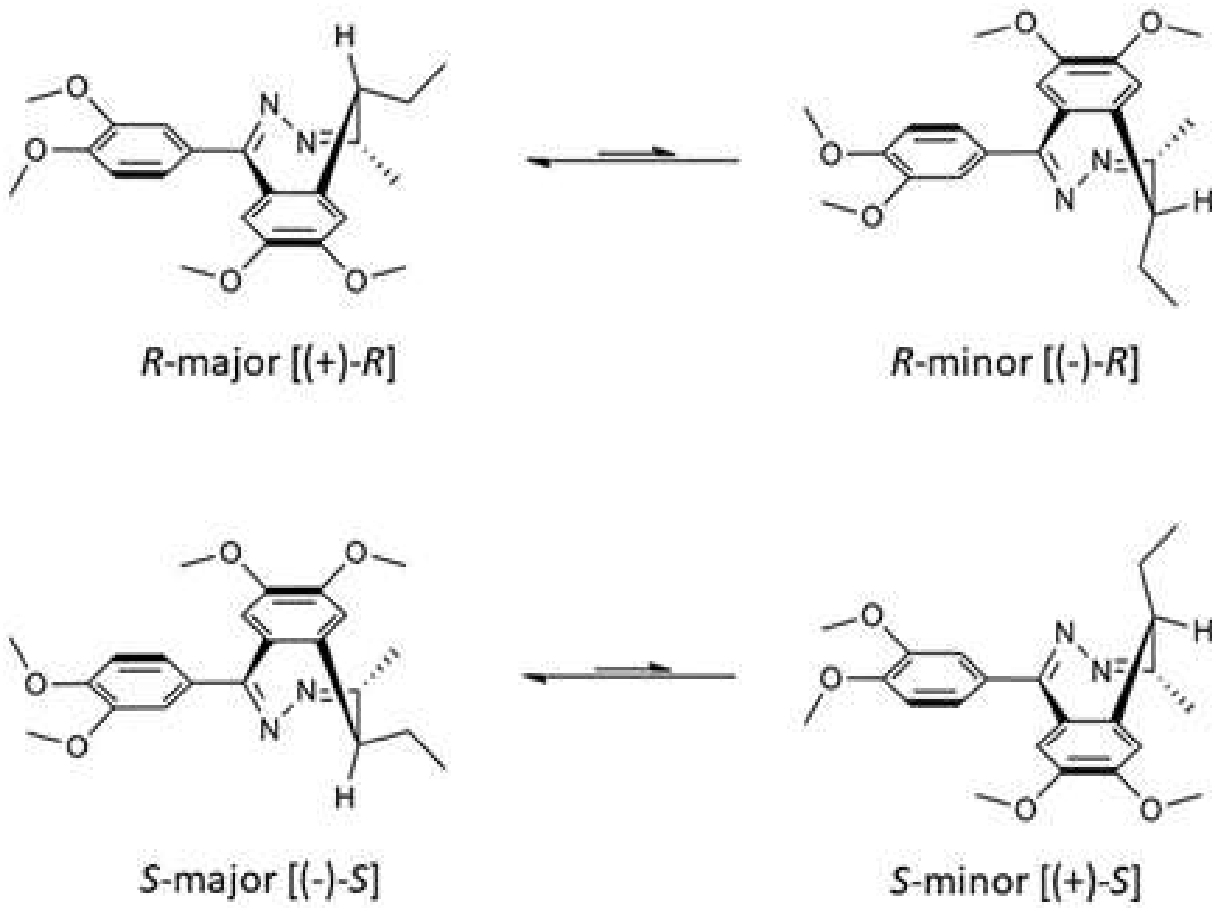

Fig-4-A: Structures and conformational that equilibria in Tofisopam enantiomers

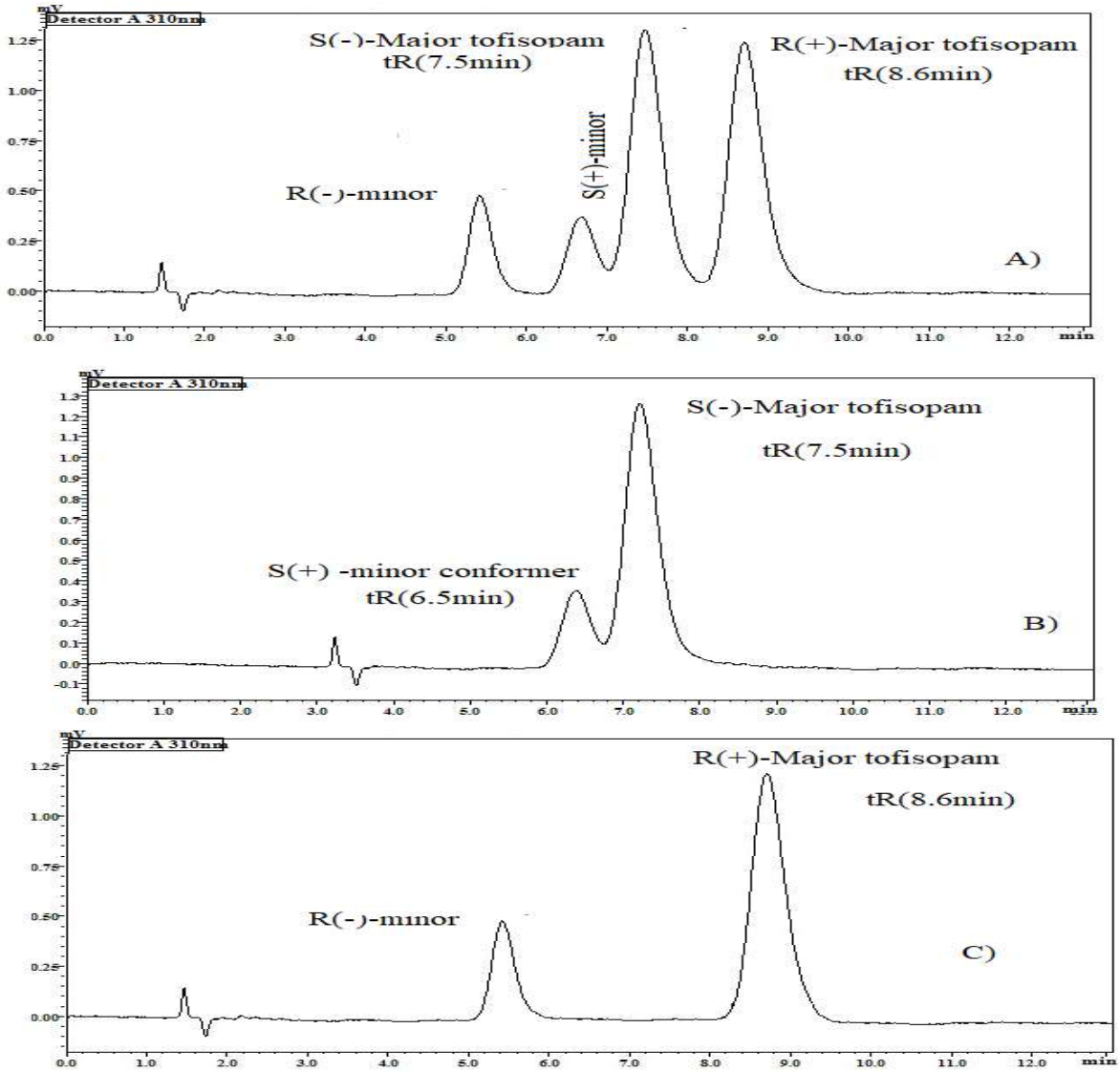

Fig-4-B: Structures and conformational that equilibria in Tofisopam enantiomers

Table-1: Calibration Data for S(-)-Tofisopam

Sl. No.	Concentration (µg/mL)	Peak Area
1	0	0
2	0.5	3314
3	1	6182
4	2	12342
5	4	23668
6	8	48397

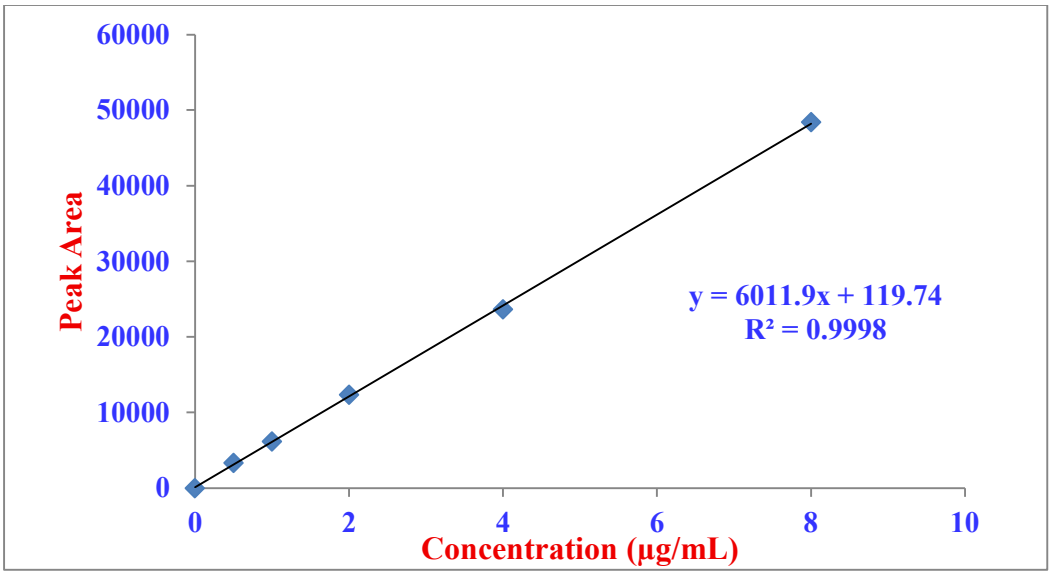

Fig-5: Standard graph for S-Tofisopam

Table 2: Calibration Data for R(+)-Tofisopam

Sl. No.	Concentration (µg/mL)	Peak Area
1	0	0
2	0.5	3378
3	1	6224
4	2	12178
5	4	24971
6	8	48971

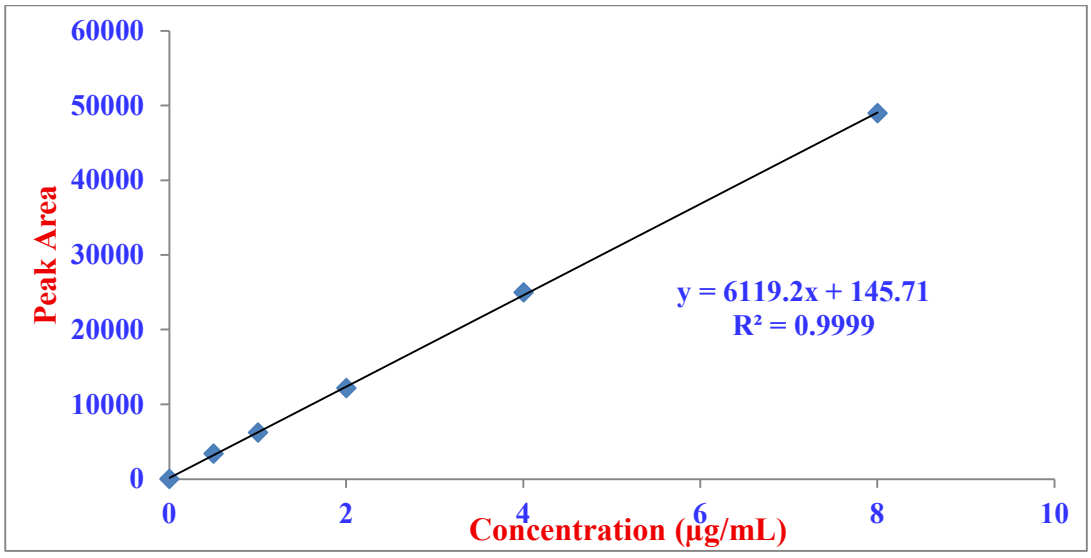

Fig-6: Standard graph for R-Tofisopam

Table-3: Recovery studies

Enantiomer	Level of Recovery (%)	Amount Added (µg/mL)	%Recovery (mean ± SD)
S-(-) Tofisopam	50	1	101.15 ± 0.22
	100	2	98.25 ± 0.25
	150	3	102.35 ± 0.28
R-(+) Tofisopam	50	1	100.75 ± 0.20
	100	2	101.15 ± 0.23
	150	3	99.50 ± 0.27

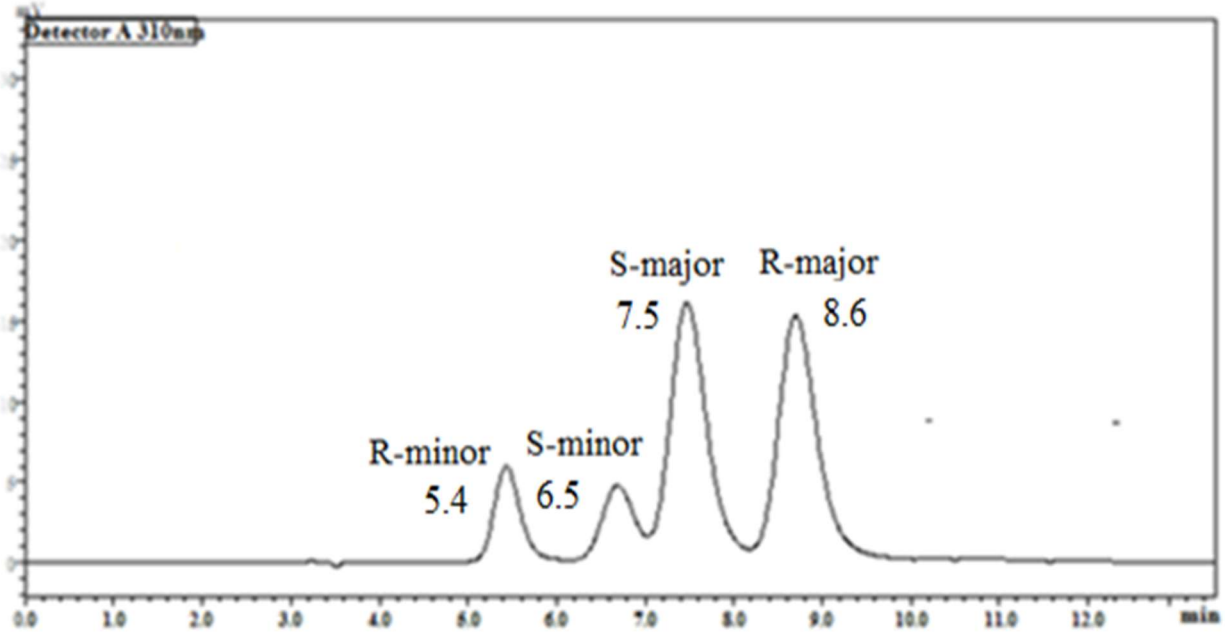

Fig-7: Enantiomeric separation of tofisopam was achieved using a Chiralcel OJ-H column (250 mm × 4.6 mm, 5 µm). The polar organic mode (POM) mobile phase consisted of MeOH/IPA and DEA in a 95:5:0.1 (v/v/v) ratio, with a flow rate set at 0.5 mL/min.

3.2. Analytical Method validation

The developed chiral-UFLC method proved suitable for the analysis of tofisopam enantiomers. System suitability was confirmed through six consecutive injections, with key parameters including retention time (tR), theoretical plates (N), tailing factor (T), and resolution (Rs) summarized in (Table-1). The good solubility of tofisopam in the mobile phase contributed to consistent chromatographic performance. Complete baseline separation of all four stereoisomers was achieved on the Chiralcel OJ-H column, with retention times of 5.4 and 6.5 minutes for minor conformers, and 7.5 and 8.6 minutes for the major enantiomers.

Calibration curves for both enantiomers over 0.5–8 µg/mL showed excellent linearity with correlation coefficients (r^2) of 0.999 (Figure 4). Intra- and inter-day precision (%RSD) at three concentration levels were below 2%, confirming method reliability (Table-3). Recovery

studies demonstrated satisfactory accuracy, with percent recoveries ranging from 98.25 to 102.35% for S-(-)-tofisopam and 99.5 to 101.15% for R-(+)-tofisopam. The LOD and LOQ were found to be 0.25 µg/mL and 0.44 µg/mL for S-(-)-tofisopam, and 0.31 µg/mL and 0.48 µg/mL for R-(+)-tofisopam, respectively. Specificity testing confirmed no interference from blank, excipients, or impurities at the retention times of the analytes. Robustness testing revealed minimal variation upon deliberate changes in flow rate, wavelength, and injection volume. Compared to previously reported methods, the proposed UFLC method provides shorter retention times, excellent resolution (Rs > 3), and high reproducibility, making it suitable for routine enantiomeric quantification of tofisopam in pharmaceutical formulations.

Table-4: System Suitability Results

Compound	Retention time (tR, min)	Theoretical plates (N)	Tailing factor (T)	Resolution (Rs)
R-(-)-tofisopam (minor conformer)	5.4 ± 0.03	–	–	–
S-(+)-tofisopam (minor conformer)	6.5 ± 0.04	–	–	–
S-(-)-tofisopam (major enantiomer)	7.5 ± 0.05	5554 ± 23	1.206 ± 0.01	–
R-(+)-tofisopam (major enantiomer)	8.6 ± 0.06	5976 ± 26	1.22 ± 0.02	3.59 ± 0.05

Table-5: Validation parameters of Tofisopam enantiomers

Enantiomer	S-(-)-Tofisopam	R-(+)-Tofisopam
Linearity range (µg/mL)	0.5–8	0.5–8
Slope	6011 ± 25	6119 ± 28
Intercept	119.7 ± 4.2	145.7 ± 5.1
Correlation Coefficient (r^2)	0.999 ± 0.001	0.999 ± 0.001
System precision (intraday, %RSD)	0.566 ± 0.03	0.488 ± 0.02
System precision (interday, %RSD)	0.683 ± 0.04	0.671 ± 0.03
Method precision (intraday, %RSD)	1.15 ± 0.06	1.158 ± 0.07
Method precision (interday, %RSD)	0.837 ± 0.05	1.257 ± 0.08
Limit of detection (LOD, µg/mL)	0.25 ± 0.02	0.31 ± 0.03
Limit of quantification (LOQ, µg/mL)	0.44 ± 0.03	0.48 ± 0.03

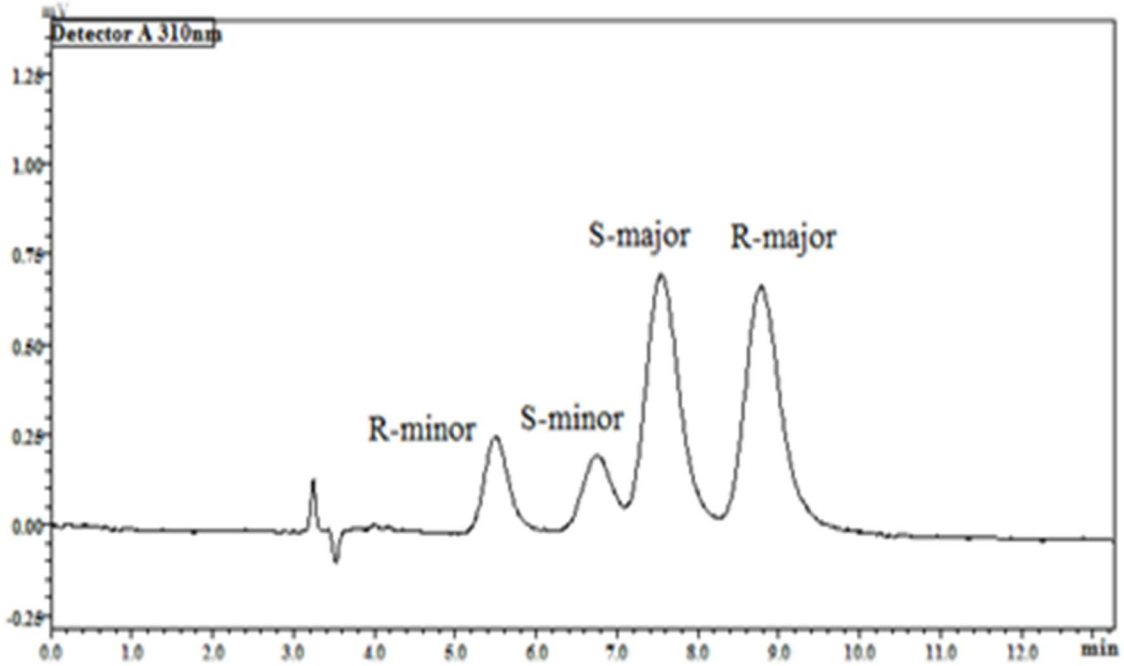

Fig-8: Chromato gramm ofracemic (±) Grandaxin Tablet

3.3. Analysis of Marketed Formulation

The validated chiral-UFLC method was successfully applied to quantify the S- and R-enantiomers of tofisopam in commercially available Grandaxin® 50 mg tablets. The assay results demonstrated excellent agreement with the labeled claim, confirming the accuracy and applicability of the developed method for routine quality control. No interference from tablet excipients was observed at the retention times corresponding to the four enantiomer peaks, ensuring method specificity. The quantification data for the enantiomers are summarized. The found quantities of S-(-)-tofisopam and R-(+)-tofisopam closely matched the declared values, with minimal variation. The results, expressed as mean ± standard deviation (SD) from three replicate determinations, indicate good precision and reproducibility of the method when applied to real pharmaceutical samples.

Table-6: Results obtained during quantification of Tofisopam enantiomers from commercially available pharmaceutical product.

Pharmaceutical product	Declared enantiomer quantity (mg)	Column1	Found enantiomeric quantity	Column2
Grandaxin® 50 mg tablet	S-TOF	R-TOF	*S*-TOF	R-TOF
	25 mg	25 mg	25.13 mg ±0.15	24.82 mg±0.10
	(6.27 mg (+)-*S* AND 18.86 mg (-)-*S)*	-	(6.26 mg (-)-*R* AND 18.56 mg (+)-*R*)	-

4. CONCLUSION

The developed chiral UFLC method, utilizing a cellulose tris-(4-methylbenzoate) stationary phase, provided rapid and efficient separation of tofisopam enantiomers. The method exhibited excellent precision, accuracy, and reproducibility across multiple trials. System suitability, linearity, and recovery studies confirmed its reliability for quantitative analysis. The limits of detection and quantification were sufficiently low to ensure sensitive measurement of both enantiomers. Robustness testing showed minimal variation under small deliberate changes in chromatographic conditions. Application to marketed formulations demonstrated accurate assay results, with no interference from excipients. The high resolution achieved allowed clear identification and quantification of major and minor enantiomers. Overall, this method is well-suited for routine enantioselective analysis of tofisopam in pharmaceutical products.

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Conflicts of Interest

The authors declare no conflicts of interest.

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