

VALIDATED CHIRAL NORMAL PHASE HPLC METHOD FOR ENANTIOSEPARATION OF ETRASIMOD

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ABSTRACT

Currently, no chiral analytical method exists for quantifying the enantiomer of Etrasimod. A swift, accurate, and valid normal-phase HPLC method was designed and validated in accordance with ICH criteria to quantify the enantiomer in both the API and drug product. Various polysaccharide chiral columns were screened under reverse-phase, polar-organic, and normal-phase circumstances. The most effective enantiomeric separation was achieved using the YMC CHIRAL ART Cellulose SJ column (25 × 0.46 cm, 5 µm), offering excellent resolution. An isocratic mode was carried out using a mobile phase containing n-hexane, methanol, ethanol, and TFA (85:10:05:0.1% v/v/v/v), with column oven temperature of 95°F, 1.0 mL/min mobile phase flow rate, and the UV detector was set at 280 nm. The method showed linearity between 0.5 and 1.875 µg/mL ($r^2 > 0.999$), with a limit of quantification and detection of 0.5 µg/mL and 0.25 µg/mL, respectively. Recovery ranged from 89% to 108%, confirming the method's precision, accuracy, and suitability for routine enantiomer quantification in quality control.

Keywords: Chiral Separation, Cellulose-SJ, ICH Guidelines, Normal Phase, Accuracy.

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INTRODUCTION

A chronic, recurrent inflammatory bowel disease that mostly affects the colon and rectum is called ulcerative colitis (UC), resulting in persistent inflammation and ulceration.¹ Patients with active UC typically experience symptoms such as bloody diarrhoea, abdominal pain, urgency to defecate, and occasionally fever, which severely impacts their quality of life.² Over time, the disease may cause major issues, such as a higher chance of colon cancer, severe dehydration, and extraintestinal manifestations affecting the eyes, joints, and skin. Etrasimod is used for the treatment of patients with significant active UC.³ As a specific regulator of the receptor of sphingosine-1-phosphate, Etrasimod contains a single chiral centre (Fig.-1) and is marketed under the trade name Velsipity.⁴ Its therapeutic effect is achieved by partially and reversibly inhibiting lymphocyte egress from lymphoid organs, thereby reducing the number of circulating lymphocytes that can migrate to the colon and cause inflammation.⁵ Although the precise mechanism remains uncertain, it is thought that inhibition of lymphocyte migration to the colon plays a key role in its efficacy.⁶ Chirality is a fundamental property of many biological molecules, such as enzymes, hormones, antibodies, and DNA, and significantly affects their pharmacological activity.⁷ Often, only one enantiomer is accountable for the intended therapeutic outcome, while the other could be hazardous or dormant. Therefore, precise analytical methods are essential for detecting and quantifying individual enantiomers in chiral active pharmaceutical ingredients (APIs) and drug products, ensuring safety and effectiveness.⁸ A comprehensive review of the literature indicates no reported chiral method for separating and quantifying enantiomers of Etrasimod using HPLC or any other analytical technique. By addressing this unmet analytical need, the study establishes a scientific foundation for future pharmaceutical evaluation, quality assurance, and regulatory applications, thereby contributing

EXPERIMENTAL

Ethanol was used to dissolve Etrasimod and its enantiomer, creating stock solutions containing 1.0 mg/mL of each. 2.5 µg/mL solutions were prepared from the stock solutions, and diluent was used to make final dilutions to achieve the necessary concentrations.

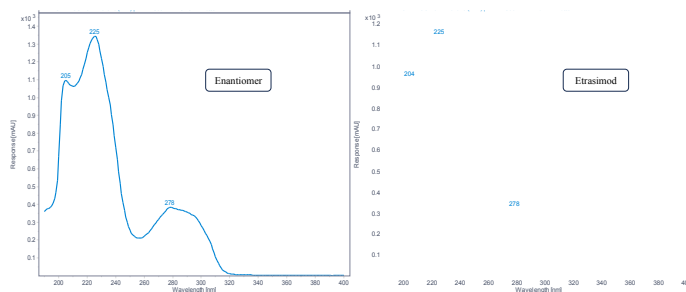


Fig.-2: UV Spectra of Enantiomer and Etrasimod

RESULTS AND DISCUSSION

Method Development

Primary Screening to Choose a Suitable Column

Polar organic (PO), reverse phase (RP), and normal phase (NP) elution conditions were used to assess a variety of coated and immobilised polysaccharide-based chiral columns. Functional groups such as cyclopentyl, trifluoromethyl, tetrahydrocyclopentaindole, and benzyl present in Etrasimod can interact with the CSP through π - π interactions, hydrogen bridges, and steric effects. Additionally, aromatic rings may insert into the CSP's chiral cavity, providing extra stabilisation of the enantiomer-CSP complex. To develop a stereoselective quantification method, the enantiomer was spiked into Etrasimod during screening experiments. Considering the compound's characteristics, TFA was added to the mobile phase as an additive.

Polar Organic Mode Screening

Polar organic solvents were used during screening in the PO mode. Four different mobile phase compositions were tested across seven distinct chiral stationary phases (CSPs). An isocratic elution approach was applied throughout the screening process. The analytes did not separate on CHIRAL ART SA, SB, SC, SZ, C Neo, and C columns. Successful enantiomer separation was achieved only with the CHIRAL ART Cellulose-SJ column using an ethanol: TFA (100:0.1%, v/v). No separation of analytes was observed on CHIRAL ART SA, SC, SZ, C Neo, and C columns using a mixture of methanol and TFA (100:0.1%, v/v). The CHIRAL ART SB column exhibited peak splitting, while only the CHIRAL ART Cellulose-SJ column achieved clear base-to-base separation of the enantiomers. The analytes were eluted on every column without separation in the acetonitrile: methanol: TFA (80:20:0.1%, v/v/v). In the composition of 2-propanol: methanol: TFA (50:50:0.1%, v/v/v), the analytes failed to separate on CHIRAL ART SA, SB, SC, SZ, C Neo, and C columns. Only the CHIRAL ART Cellulose-SJ column exhibited some degree of separation, but it was insufficient.

Reverse-Phase Mode Screening

Reverse-phase screening was performed using a combination of acetonitrile and acidic aqueous buffers (0.1% TFA or 0.1% FA in water) under a gradient program: 0/40, 10/85, 20/85, 21/40, 30/40. The first mobile phase used 0.1% TFA in water and acetonitrile, while the second replaced TFA with 0.1% FA. Analytes failed to separate on CHIRAL ART SA, SC, SJ, and SZ columns. Although CHIRAL ART SB and C Neo showed some degree of separation, it was insufficient in both mobile phases. Thus, this approach did not achieve effective resolution between Etrasimod and its enantiomer.

Normal Phase Mode Screening

Standard solvents, including n-hexane, ethanol, methanol, and 2-propanol, were used in the mobile phases. No separation of analytes occurred on CHIRAL ART SA, SC, SZ, C Neo, and C columns using n-hexane, ethanol, and TFA (80:20:0.1%, v/v/v). The CHIRAL ART SB column displayed peak splitting, while CHIRAL ART Cellulose-SJ achieved base-to-base separation, though with noticeable peak tailing. Using n-hexane, 2-propanol, and TFA (80:20:0.1%, v/v/v), analytes did not separate on CHIRAL ART SA, SB, SC, SZ, C Neo, and Cellulose-C columns. Some separation was observed on the CHIRAL ART Cellulose-SJ column. With the final mobile phase, n-hexane, 2-propanol, methanol, and TFA

(80:10:10:0.1%, v/v/v/v), no separation was observed on CHIRAL ART SA, SB, SC, SZ, C Neo, and C columns. In contrast, the CHIRAL ART Cellulose-SJ column provided improved separation and better peak shapes, making it the column of choice for subsequent optimisation experiments.

Optimisation of the Mobile Phase Combinations

Following extensive screening in PO, RP, and NP modes, CHIRAL ART Cellulose-SJ demonstrated superior separation and peak shapes compared to other columns. Numerous experiments varying mobile phase composition and additives were performed to enhance resolution. Using dual additives (DEA and TFA) in n-hexane, ethanol, and methanol (90:05:05 v/v/v) did not improve separation. Ultimately, a mobile phase of n-hexane, ethanol, methanol, and TFA (85:05:10:0.1% v/v/v/v) provided optimal enantiomeric separation, highlighting the significant influence of solvent polarity on elution and resolution.

Optimisation of Column Oven Temperature

When it comes to chiral HPLC enantiomeric separation, column temperature is crucial. Using n-hexane, ethanol, methanol, and TFA (85:10:10:0.1% v/v/v/v), trials were performed at 59°F, 77°F, and 95°F. Among these, 95°F provided the best resolution and peak symmetry, as shown in Table-1. The optimised conditions employed n-hexane: ethanol: methanol: TFA (85:05:10:0.1% v/v/v/v) at 35°C with a 1.0 mL/min mobile phase flow rate. The enantiomeric separation of Etrasimod was achieved on a cellulose-based CHIRAL ART Cellulose-SJ column, which contains Cellulose tris 4-methylbenzoate chiral selectors immobilised on silica.⁹ According to the literature, cellulose-based chiral stationary phases offer several stereoselective interactions, such as π - π interactions, dipole-dipole interactions, steric fitting, and hydrogen bonding, which play a crucial role in enantiomer identification.¹⁰

The aromatic rings and heteroatoms that exist in Etrasimod may interact variously with the chiral cavity of the chiral selector, resulting in different retention of the two enantiomers. The mobile phase consisting of n-hexane, methanol, ethanol, and TFA (85:10:05:0.1% v/v/v/v) offered the best balance of solvation and chiral recognition. The nonpolar n-hexane encourages strong interactions between the analytes and the stationary phase, whereas the polar modifiers, ethanol and methanol, control elution strength and adjust selectivity.^{11,12} The addition of TFA reduces the ionisation of the basic group present in Etrasimod, increasing peak shape and resolution.¹³ The effective separation of pharmaceutical enantiomers has been widely documented in the literature using chiral stationary phases made of cellulose and alcohol modified hexane mobile phases.

Method Validation

System Suitability Test

Six duplicates of the standard solution were injected to assess the system's suitability. Table-1 presents the results, including resolution (RS), tailing factors (TF), and theoretical plate counts (TP). The percentage RSD was 0.039.

Table-1: Findings of Different Studies

Parameter	Conditions	Resolution	Retention time(min)		Tailing Factor		Theoretical plates	
			A	B	A	B	A	B
Temperature	15°C	3.550	12.640	10.400	1.37	1.40	5001	5777
	25°C	3.660	11.990	9.990	1.35	1.34	6280	6783
	35°C	3.900	10.650	9.060	1.25	1.26	9382	9400
System suitability	Ideal	3.460	11.104	9.707	1.135	1.163	10876	10335
Robustness	Ideal	3.472	11.093	9.682	1.120	1.135	10685	10178
	0.8 mL/min	3.725	13.892	12.110	1.112	1.157	12129	11498
	1.2 mL/min	3.257	9.230	8.060	1.129	1.144	9471	9048
	30 °C	3.670	12.117	10.432	1.143	1.173	9706	9580
	40°C	3.199	10.229	9.038	1.102	1.149	11069	10321

Specificity

The enantiomer, API, and blank were present when the newly discovered method's specificity was assessed. Stereospecificity was confirmed as no interference from blank peaks was seen at the RT of the enantiomer or API. Figure-3 shows a standard chromatogram of Etrasimod with a 0.15% enantiomer spike, alongside the blank, demonstrating that the blank did not affect the primary peaks.

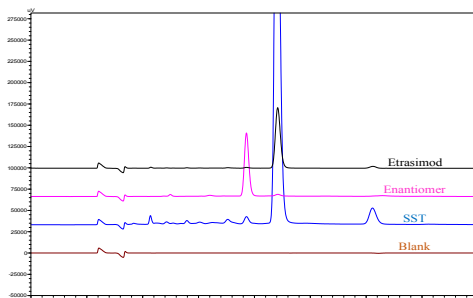


Fig.-3: Typical Overlay Chromatograms for the Specificity Study

Linearity

By creating a calibration curve for the sample, we evaluated linearity. Six concentrations, from the LOQ level to 1875 ng/mL, were prepared and injected in triplicate. Linearity was assessed using the technique of least squares, yielding coefficients of correlation (r^2) of 0.9998 for Etrasimod API and 0.9994 for its enantiomer. The corresponding calibration plots are shown in Fig.-4.

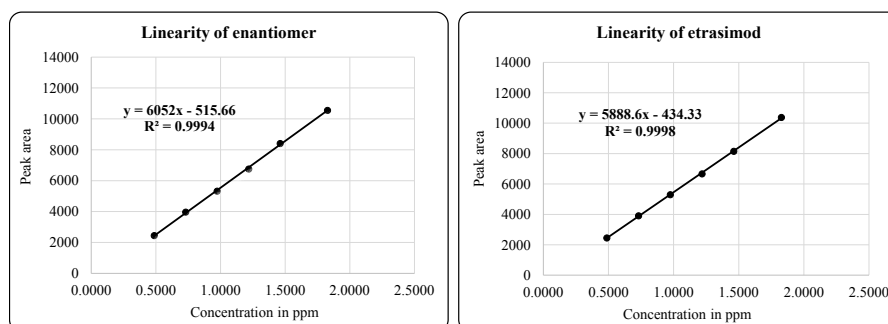


Fig.-4: The Linearity Graphs for Etrasimod and its Enantiomer

Accuracy

The accuracy (0.15% to 1 mg/mL of sample) of the enantiomer was examined in triplicate at four different concentrations (0.5, 0.75, 1.5, and 2.25 $\mu\text{g/mL}$), spanning from the LOQ to 150% of the target concentration. Recovery percentages were calculated at each level, with values ranging from 89% to 107% for the enantiomer in the Etrasimod sample. Detailed results are presented in Table-2.

Table-2: Percentage Recovery Values for the Enantiomer

Accuracy level	Percentage recovery			Average recovery (%)
	Prep-01	Prep-02	Prep-03	
LOQ	105.75	98.19	98.90	100.95
50%	104.37	103.58	98.99	102.31
100%	105.89	107.08	108.81	107.26
150%	85.75	92.53	89.53	89.27

Method Precision

With various analysts conducting the assessments, the intermediate (inter-day) precision of the enantiomer's retention time and peak area was evaluated in order to ascertain the method's precision. Six replicate injections of a 0.15% test concentration (1.0 mg/mL) of the enantiomer spiked into Etrasimod

were analysed to assess reproducibility. Repeatability showed a %RSD of 0.43, while inter-day precision, evaluated with six replicates, yielded a %RSD of 1.92.

LOQ and LOD

The LOD and LOQ of the enantiomer were ascertained by injecting diluted samples of known concentrations based on S/N ratios of 3:1 and 10:1, respectively. The enantiomer's LOD was determined to be 0.25 µg/mL, while its LOQ was set at 0.5 µg/mL.

Robustness

By purposefully changing chromatographic parameters, such as column oven temperature ($\pm 5^{\circ}\text{C}$) and flow rate (± 0.2 mL/min), the method's resilience was assessed. The Etrasimod–enantiomer mixture was injected under each condition, and retention time, resolution, theoretical plate count, and tailing factor were assessed. All parameters remained within acceptable limits, showing the method's robustness. Detailed results are shown in Table-1. To the best of our knowledge, this is the first report describing the chiral development and validation of a chromatographic method for the analysis of Etrasimod. No previously published studies have addressed its systematic separation optimisation or analytical validation under the present conditions. The novelty of this work lies in the successful development of a reliable, reproducible analytical approach that enables accurate quantification and effective separation. This study, therefore, contributes new scientific knowledge to the field of analytical chemistry by introducing a validated method that can serve as a reference for further investigations, stability studies, and quality control assessments of this molecule. This research significantly contributes to Sustainable Development Goal 3 (Good Health and Well-being) by supporting the assurance of pharmaceutical quality and safety. The development of a validated and reliable analytical method ensures accurate quantification and detection, thereby minimising the risk of substandard or ineffective medicinal products. By strengthening analytical reliability in pharmaceutical quality control, the present study promotes safer therapeutic outcomes and enhances public health protection. The method established in this work provides a scientific foundation for regulatory compliance and industrial application, thereby reinforcing the sustainable healthcare system.

CONCLUSION

A new, thoroughly verified chiral HPLC technique was effectively created for the simultaneous measurement of Etrasimod enantiomers. Using the CHIRAL ART SJ (25×0.46 cm, $5\ \mu\text{m}$) immobilised polysaccharide column in isocratic elution mode. The method demonstrated excellent resolution, accuracy, precision, linearity, specificity, and sensitivity. It is robust under varied chromatographic conditions and was capable of reliably detecting and quantifying enantiomeric impurities in Etrasimod. Given its simplicity and reproducibility, this method is appropriate for regular use in quality control laboratories to ensure the safety, efficacy, and purity of bulk drug substances and formulations.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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