



OPEN Possibilities and limitations of computer assisted chiral HPLC method development for ozanimod on polysaccharide based chiral stationary phases

Elek Ferencz^{1,6}, Zoltán-István Szabó^{1,2,6}, Arnold Zöldhegyi³, Gergely Dombi^{4,5}, Gergely Molnár^{4,5}, Máté Dobó^{4,5}, Erzsébet Varga¹, Imre Molnár³ & Gergő Tóth^{4,5,6}✉

In this study, a direct HPLC method was developed to determine the enantiomeric purity of the immunomodulatory drug, ozanimod. A systematic method development process was followed, incorporating risk assessment, identification of critical analytical procedure parameters, initial screening of stationary phases, and software-assisted optimization of method parameters. Eight different polysaccharide-based chiral columns were selected to assess chiral separation of enantiomers under polar organic elution mode. The most promising results were obtained using a methanol:2-propanol mixture on the amylose-based Chiralpak AD column. Following this, systematic modeling was conducted using DryLab software to optimize method conditions, including isocratic eluent composition, temperature, and flow rate. Baseline separation was achieved within fifteen minutes using the optimized parameters: Chiralpak AD column thermostated at 10 °C, and a mobile phase of methanol:2-propanol: diethylamine, 70:30:0.1 (v/v/v %), delivered at a flow rate of 0.8 mL/min. The developed method was validated according to current guidelines and *in silico* robustness testing was conducted to determine tolerance limits for critical separation parameters and their impact on enantioresolution. Our findings demonstrate the utility of DryLab, typically employed for reversed-phase achiral separations, in optimizing chiral methods even in polar organic mode. Limitations of the selected approach the development of chiral separation methods are also highlighted.

Keywords Ozanimod, Chiral separation, Polysaccharide-type chiral column, AQbD, DryLab, Computer-assisted method development

Ozanimod emerges in the clinical landscape as one of the promising candidates for the treatment of multiple sclerosis due to the more patient-friendly therapy by its oral administration route and the unique capability to modulate immune response^{1–3}. Chemically, ozanimod is a sphingosine analogue, and its engineered structure allows effective interactions with the S1P receptor system, eliciting the desired pharmacological responses, while minimizing off-target effects^{2,4}.

Ozanimod contains a singular chiral carbon atom, implying the existence of two enantiomeric forms, namely *S*-ozanimod (commonly known as ozanimod) and *R*-ozanimod (Fig. 1).

In therapeutic applications, the more effective *S*-enantiomer is employed. Consequently, regulatory authorities mandate the determination of the *R*-antipode in ozanimod sample as part of the necessary assessments^{5–7}. Despite this, current literature reveals a missing context concerning enantiopurity determination of ozanimod. Only two articles deal with the chiral separation of ozanimod, one use normal-phased mode on Chiralpak AS column⁸, while the other use PO mode on amylose tris(3,5-dimethylphenylcarbamate) column⁹. Moreover, there are few articles and some patents available regarding the enantioselective synthesis of the drug^{8,10–12}. This discrepancy

¹George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Targu Mures, Romania. ²Sz-imfidum Ltd, Lunga 525401, Romania. ³Molnár-Institute for Applied Chromatography, Berlin, Germany. ⁴Department of Pharmaceutical Chemistry, Semmelweis University, Hőgyes E. u. 9, Budapest 1092, Hungary. ⁵Center for Pharmacology and Drug Research & Development, Semmelweis University, Budapest, Hungary. ⁶Elek Ferencz and Zoltán-István Szabó contributed equally to this work. ✉email: toth.gergo@semmelweis.hu

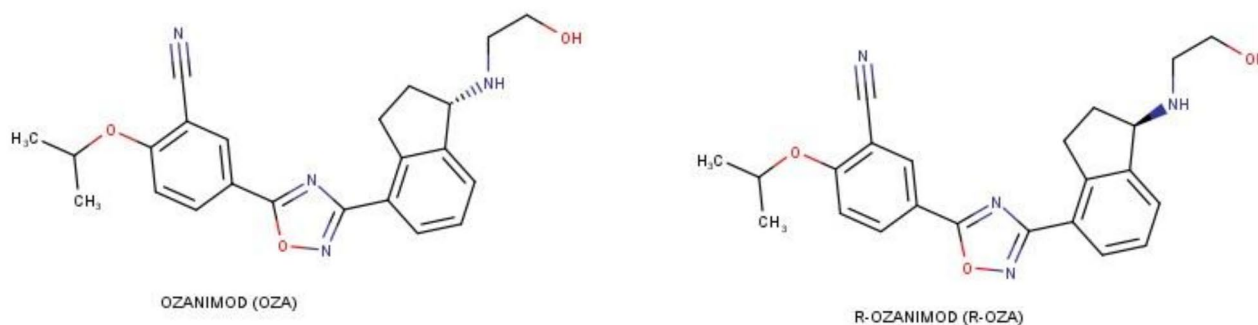


Fig. 1. Structures of ozanimod (eutomer) and *R*-ozanimod (distomer).

highlights a research gap in the field, emphasizing the need for investigations into chiral separation techniques for ozanimod, ensuring accurate determination of enantiomeric purity, which is crucial for therapeutic efficacy and regulatory compliance.

Today, a variety of methods are utilized for chiral analysis, including capillary electrophoresis, gas chromatography, high performance liquid chromatography (HPLC), and supercritical fluid chromatography (SFC). Among them, direct HPLC enantioseparation in combination with a chiral stationary phase (CSP) is the gold standard in the field^{13,14}. The most popular and effective CSPs are commonly based on polysaccharides^{15–17}. These columns also offer versatility, as they can be employed in normal-phase (NP), reversed-phase (RP)¹⁸, and polar organic (PO) modes^{19,20}. However, in some cases these CSPs may offer chemo- and stereoselectivity for simultaneous separation of achiral and chiral related substances. Despite being toxic and environmentally unfriendly, many official compendial enantioselective methods still up to this day use NP mode. However, numerous studies have shown that PO mode, using simple mobile phase compositions, such as neat alcohols or acetonitrile (ACN), can be equally effective or even surpass NP, offering several advantages, such as environmental friendliness, shorter run times and improved peak shapes. Not only neat eluents but mixtures of methanol (MeOH) and 2-propanol (IPA) or ACN and alcohols are commonly used in PO mode to alter peak shape, selectivity, and enantiomer elution order (EEO)^{21–23}. One of the interesting effects of PO mode is the hysteresis of retention times and selectivity, that cause sometimes even changes of EEO^{9,24–26}. In some cases, these polysaccharide-based CSPs used in PO mode can offer chemo- and stereoselectivity for simultaneous separation of achiral and chiral related substances^{27–29}.

It is crucial to emphasize that the development of chiral separations often relies on conventional methods, involving identification of suitable chiral selectors, followed by a single optimization step of method parameters through trial and error or the one-factor-at-a-time approach. This traditional or empirical development strategies can result in prolonged processes and suboptimal outcomes. In contrast, multivariate methodologies have demonstrated their effectiveness in overcoming these limitations. By employing experimental designs, they allow for the simultaneous evaluation of critical method parameters while monitoring their impact on selected performance indicators. These comprehensive approaches promote a deeper understanding with fewer experimental iterations, facilitating the identification of relationships between variables^{30,31}.

Hence, it is not surprising that current regulatory guidelines such as International Council of Harmonization (ICH) Q14³² and the United States Pharmacopeia (USP) General Chapter <1220>³³ also foster the integration of Analytical Quality by Design (AQbD) principles within the life cycle framework. This integration aims to enhance method robustness throughout the method's intended use. One significant advantage of AQbD lies in its capacity to transition from a rigid workpoint to more flexible methodologies through the incorporation of defined tolerances within a multidimensional Design Space. This integration enhances decision-making processes rooted in risk and knowledge, thereby helping to prevent exhaustive investigations into out-of-specification occurrences.

Currently, three main groups of chromatography modeling software have been made commercially available. The software of the first group uses a pure empirical “black-box” approach to uncover patterns and relationships of a set of experimental data. To this group belong tools like Fusion QbD, JMP, MODDE, Statistica, Design-Expert and others^{20,27–29}. The second group uses molecule structures along with physicochemical properties and specific molecule descriptors sometimes combined with experimental data to predict expected chromatographic behavior of compounds. Examples are ChemSpider, ChromSword, EluEx, PALLAS and others. The third class uses a mechanistic design to connect experimental data points, i.e., one or more chromatographic variables with modeling responses, typically critical resolution (software packages like DryLab, ACD Labs, and others)^{18,32}.

Molecule-structure based modeling may prove inadequate, particularly when dealing with structural isomers, where identical molecular formulas result in identical retention times³³. Presently, statistical-based development predominates in chiral chromatography however, it is often deemed less efficient in terms of resource utilization and inattentive use of any “black-box” tool may result in oversight of some chromatographically relevant parameters. As stated in USP <1220> “When available, mechanistic models can be used to understand the effect of procedure parameters on performance. The use of mechanistic models can reduce experimental work and provide a reliable estimate of the behavior of the analytes of interest”³⁴.

Bearing this in mind, we selected one of the most established, chromatography-based modeling tools (DryLab) to investigate modeling benefits regarding time-, and performance-improvements of chiral separations in PO mode. It is worth mentioning that this tool was originally designed for modeling RP-systems but has demonstrated versatility over its 38-year history^{35,36}, spanning various chromatographic applications including separation of small, and large molecules in various modes of chromatography such as ion-pairing reversed phase (IP-RP)³⁷, ion-exchange (IEX)^{38,39}, hydrophobic interaction (HIC)⁴⁰ and hydrophilic interaction chromatography (HILIC)³², and most recently SFC⁴¹. DryLab has demonstrated success in optimizing the enantioseparation of racemic 4-dinitrophenyl amino acids using quinine carbamate-type chiral stationary phase with water-based mobile phase⁴² and atropoisomers⁴³ on a derivatized β -cyclodextrin bonded stationary phase using reversed-phase mode. In a previous study, we discussed how a general modeling approach can be applied to separate achiral analytes with closely similar structures in reversed-phase mode using chiral stationary phases¹⁸. However, it is important to acknowledge that in such cases, the prediction accuracy of virtual separation models, particularly for polysaccharide CSPs, may be somewhat lower. For instance, a few years ago, Wagdy successfully tested DryLab's applicability on several CSPs containing immobilized antibiotic groups like teicoplanin and vancomycin. However, modeling on a polysaccharide phase (amylose carbamate) was proven unfeasible⁴⁴. Nevertheless, it can remain an effective modeling tool in refining method selectivity even in those scenarios.

Our study aimed to revisit modeling possibilities for chiral separation by developing an enantioselective method for determination of the enantiomeric purity of ozanimod, following the principles outlined in the current ICH Q14 guideline and by leveraging the most recent modeling options provided by DryLab.

Materials and methods

Materials

Ozanimod and *R*-ozanimid were from Egis Pharmaceuticals PLC (Budapest, Hungary). Gradient grade HPLC eluents (MeOH, IPA, ACN) were obtained from Merck KGaA (Darmstadt, Germany). Lux i-Amylose-1 [Amylose tris(3,5-dimethylphenylcarbamate)], Lux Amylose-2 [Amylose tris(5-chloro-2-methylphenylcarbamate)], Lux Cellulose-1 [Cellulose tris(3,5-dimethylphenylcarbamate)], Lux Cellulose-2 [Cellulose tris(3-chloro-4-methylphenylcarbamate)], Lux Cellulose-3 [Cellulose tris(4-methylbenzoate)], Lux Cellulose-4 [Cellulose tris(4-chloro-3-methylphenylcarbamate)] (150 × 4.6 mm, particle size 5 μ m) were obtained from Phenomenex (Torrance, CA, USA). Chiralpak AD [Amylose tris(3,5-dimethylphenylcarbamate)] and Chiralcel OD [Cellulose tris(3,5-dimethylphenylcarbamate)] (250 × 4.6 mm; particle size 10 μ m) were the products of Daicel Corporation (Tokyo, Japan).

LC-UV analysis and software

LC-UV analyses were carried out on a JASCO HPLC system equipped with a JASCO PU-2089 Plus quaternary gradient pump, AS-4050 autosampler, MD-2010 Plus diode array detector and CO-2065 Plus column oven (Jasco Corporation, Tokyo, Japan). For the instrument control and data processing ChromNAV (Ver. 2.0, Jasco Corporation, Tokyo, Japan, URL: <https://jascoinc.com/products/chromatography/hplc/hplc-software/>) was used. Acquired experimental results were processed and exported into standardized AIA/ANDI-formats (*.cdf). Retention modeling was accomplished with DryLab 4 (Ver. 4.5, URL: <https://molnar-institute.com/drylab/>) modeling software (Molnár-Institute, Berlin, Germany). Modeling steps included experimental design (DoE), peak-tracking, extensive design space studies and in silico robustness testing.

All samples were dissolved in pure MeOH, stock solutions were prepared at 1 mg/mL concentrations, further dilutions were made using pure MeOH as sample solvent. The ratio between diastomer-eutomer was 1:3 during the preliminary experiments (100 μ g/mL vs. 300 μ g/mL). Injection volume was 2.0 μ L, each experiment was replicated 3 times. The final test solution of ozanimod for validation and method applicability testing was 1000 μ g/mL. The impurity level percentages were calculated relative to this concentration. To ensure proper run-to-run re-equilibration of the chromatographic system, we flushed the column with 10 effective column volumes (CV) prior to injection. Throughout the preliminary study column temperature set to 25 °C. For Lux columns the flow rate was set to 0.5 mL/min, while for Daicel columns, it was programmed to 0.7 mL/min. The backpressure was under 130 bar for all cases. For the analyses, detection wavelength was uniformly set to 270 nm.

Results and discussion

Primary data collection and defining the analytical target profile

In accordance with the ICH Q14 guideline, the first step of the method development is collecting prior data about the compounds and formulation of the Analytical Target Profile (ATP) including clear method objectives and performance criteria.

Analyzing previous literature, Németh and Horváth previously investigated this compound as a model substance in PO mode. Utilizing an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase, they effectively achieved enantioseparation⁹, while Cianferotti et al. determined the enantiomer ratio of ozanimod enantiomers with a NP method using Chiralpak AS-H column with hexane:2-propanol:DEA mixture⁸.

Ozanimod has lipophilic properties ($\log P = 3.73$), with a pH-sensitive basic secondary amine embedded within its structure ($pK_a = 8.96$).

From a chromatographic perspective, employing a high pH with a basic additive is necessary to minimize the ionization of the secondary amine. Considering the $\log P$ value and available literature, it is advisable to dissolve ozanimod in methanol.

With all of this in view, we defined our ATP goals: The enantiomeric purity testing method should accurately quantify *R*-ozanimod in the ozanimod sample, meeting accuracy and precision requirements of $100.0\% \pm 3.0\%$ and $\leq 2.0\%$, respectively. It should also be able to allow quantification of the enantiomeric impurity of at least

0.05% in ozanimod samples, while also ensuring adequate peak symmetry (T_f in the usual range of 0.8–2.0). Additional expectations included short run time, preferably less than 20 min, fixed EEO (distomer eluting before the eutomer) and resolution values (R_s) > 2.0 even if the method is in routine use (established robustness). Building upon these considerations, chiral HPLC employing polysaccharide columns under PO mode has been selected as the preferred technique. Furthermore, environmental concerns were taken into consideration, with the PO mode chosen for its eco-friendliness, avoiding toxic eluents such as hexane.

Risk assessment (RA) and identification of potential critical analytical procedure parameters (APPs)

As a subsequent step of method development, in accordance with the ICH Q14 guideline the risk assessment-based identification of the APPs is necessary⁴⁵.

Ishikawa diagrams are typically well-suited for categorizing APPs to select critical parameters⁴⁶. In our scenario, parameters concerning the separation system—specifically, the type of stationary phase and applied conditions—have been identified as critical APPs. The resulting heat map from the risk matrix outlined in Supplementary Table 1 further highlights the risks associated with column chemistry and eluent composition. In this representation, red denotes situations of higher risk, necessitating further investigation, analysis, or attention. Conversely, green, and yellow boxes indicate a low to medium impact on performance criteria, accompanied by a reduced likelihood of occurrence. In our case, both the identified APPs and the critical APPs with potential impact on analytical procedure attributes (APA) are shown in Fig. 2.

Initial stationary phase selection

Previous RAs have underscored the critical significance of selecting the appropriate stationary phase. However, due to the complex separation processes in chiral chromatography, it is not possible to reliably predict the accurate resolving capability of a chiral selector. Consequently, preliminary screening studies are typically necessary. To evaluate the enantioseparation capabilities of various stationary phases, we selected eight chiral columns (Chiralpak AD, Chiralcel OD, Lux i-Amylose-1, Lux Amylose-2, Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3, and Lux Cellulose-4) and conducted some initial experiments on them using MeOH ACN, and IPA as mobile phases, supplemented with 0.1% DEA as a basic additive to control the ionization of ozanimod.

Moreover, DEA modified IPA-MEOH mixtures were also applied, as a potential chance to improve the resolution. Throughout the preliminary study, we maintained other parameters fixed, with the column temperature set to 25 °C. For Lux columns the flow rate was set to 0.5 mL/min, while for Daicel columns, it was programmed to 0.7 mL/min, irrespective of the chosen eluent. The results of the preliminary experiments are summarized in Table 1, and representative chromatograms depicted in Fig. 3.

These results indicated that except for the Lux i-Amylose-1 column, all chiral selectors show enantio-recognition towards ozanimod enantiomers, however in some cases the resolution values are low (Lux amylose-1, Chiralcel OD). Initial resolution values far exceeded the ATP specified minimum of 2.0 in some cases. Notably, on the

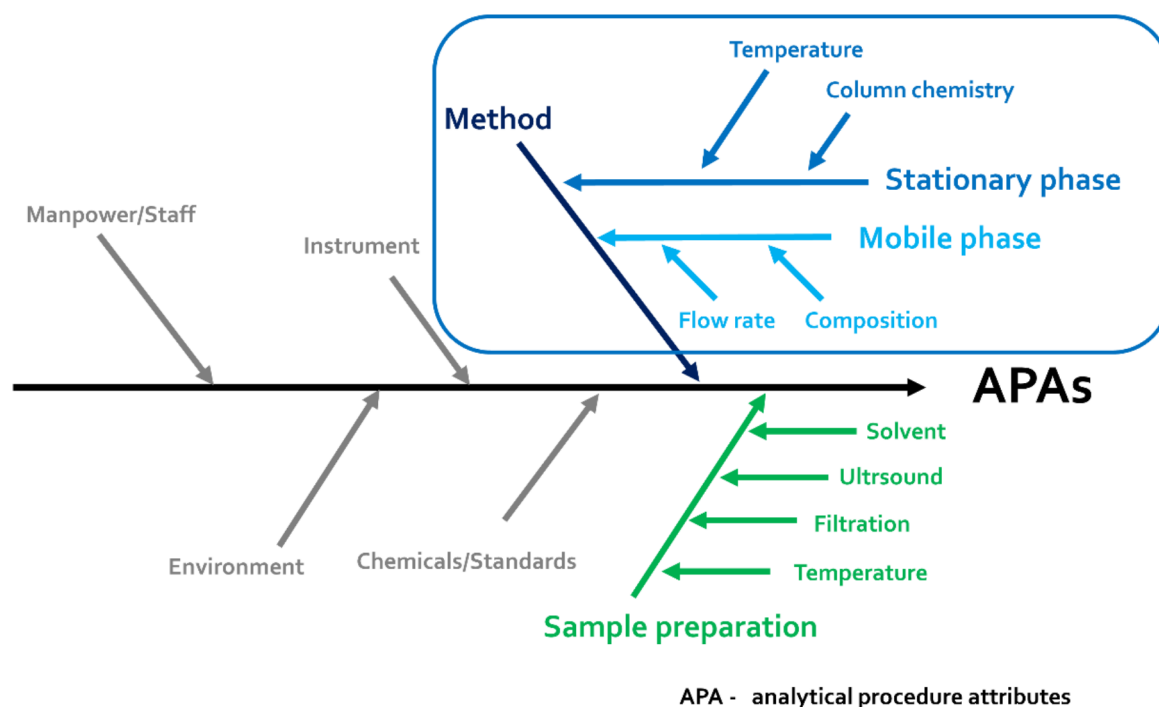


Fig. 2. Risk assessment procedure illustrated on Ishikawa diagram presenting APPs and critical APPs (encircled) which have potential impact on method performance (expressed in analytical procedure attributes).

Column	Mobile phase*	k_1	k_2	R_s	EEO
Lux cellulose-1	MeOH	1.16	–	–	
	MeOH: IPA 70:30	1.07	–	–	
	MeOH: IPA 50:50	1.02	–	–	
	MeOH: IPA 30:70	1.06	–	–	
	IPA	1.40	–	–	
	ACN	0.88	0.98	0.32	S < R
Lux cellulose-2	MeOH	1.88	2.23	2.52	S < R
	MeOH: IPA 70:30	1.54	1.87	2.11	S < R S < R
	MeOH: IPA 50:50	1.46	1.78	1.86	S < R
	MeOH: IPA 30:70	1.58	1.89	1.43	S < R
	IPA	2.51	2.97	0.88	S < R
	ACN	2.59	3.20	3.23	S < R
Lux cellulose-3	MeOH	1.08	1.22	1.21	S < R
	MeOH: IPA 70:30	1.01	1.11	0.99	S < R
	MeOH: IPA 50:50	0.93	1.02	0.73	S < R
	MeOH: IPA 30:70	0.85	–	–	
	IPA	1.67	–	–	
	ACN	0.47	–	–	
Lux cellulose-4	MeOH	1.25	1.62	3.60	S < R
	MeOH: IPA 70:30	1.20	1.51	3.18	S < R
	MeOH: IPA 50:50	1.12	1.39	2.67	S < R
	MeOH: IPA 30:70	1.05	1.28	2.12	S < R
	IPA	2.01	2.78	1.67	S < R
	ACN	1.94	2.14	1.23	S < R
Chiralcel OD	MeOH	1.97	2.09	0.55	R < S
	MeOH: IPA 70:30	1.86	–	–	
	MeOH: IPA 50:50	1.82	–	–	
	MeOH: IPA 30:70	1.92	–	–	
	IPA	2.12	–	–	
	ACN	1.05	–	–	
Lux i-amylose-1	MeOH	0.85	–	–	
	MeOH: IPA 70:30	0.58	–	–	
	MeOH: IPA 50:50	0.54	–	–	
	MeOH: IPA 30:70	0.56	–	–	
	IPA	0.42	–	–	
	ACN	1.33	–	–	
Chiralpak AD	MeOH	2.30	–	–	
	MeOH: IPA 70:30	1.65	1.96	2.10	R < S
	MeOH: IPA 50:50	1.52	1.77	1.51	R < S
	MeOH: IPA 30:70	1.50	1.65	1.10	R < S
	IPA	0.57	–	–	
	ACN	1.97	2.48	0.51	S < R
Lux amylose-2	MeOH	0.01	–	–	
	MeOH: IPA 70:30	0.03	–	–	
	MeOH: IPA 50:50	0.05	–	–	
	MeOH: IPA 30:70	0.14	–	–	
	IPA	0.52	–	–	
	ACN	1.34	2.20	2.03	R < S

Table 1. The results of preliminary screening with the retention factor of the ozanimod enantiomers (k_1 and k_2), resolution (R_s) and enantiomeric elution order (EEO). *With 0.1% DEA.

Lux Cellulose-2 column with ACN and MeOH (Fig. 3A and B), and the Lux Cellulose-4 column with methanol, resolution values reached as high as $R_s = 3.6$ (Fig. 3C). However, with cellulose-based columns—except for Chiralcel OD with MeOH—the elution order enantiomer (EEO) was found to be not ideal, as the distomer tended to elute first. It is important to acknowledge that despite this undesired EEO, the high-resolution values

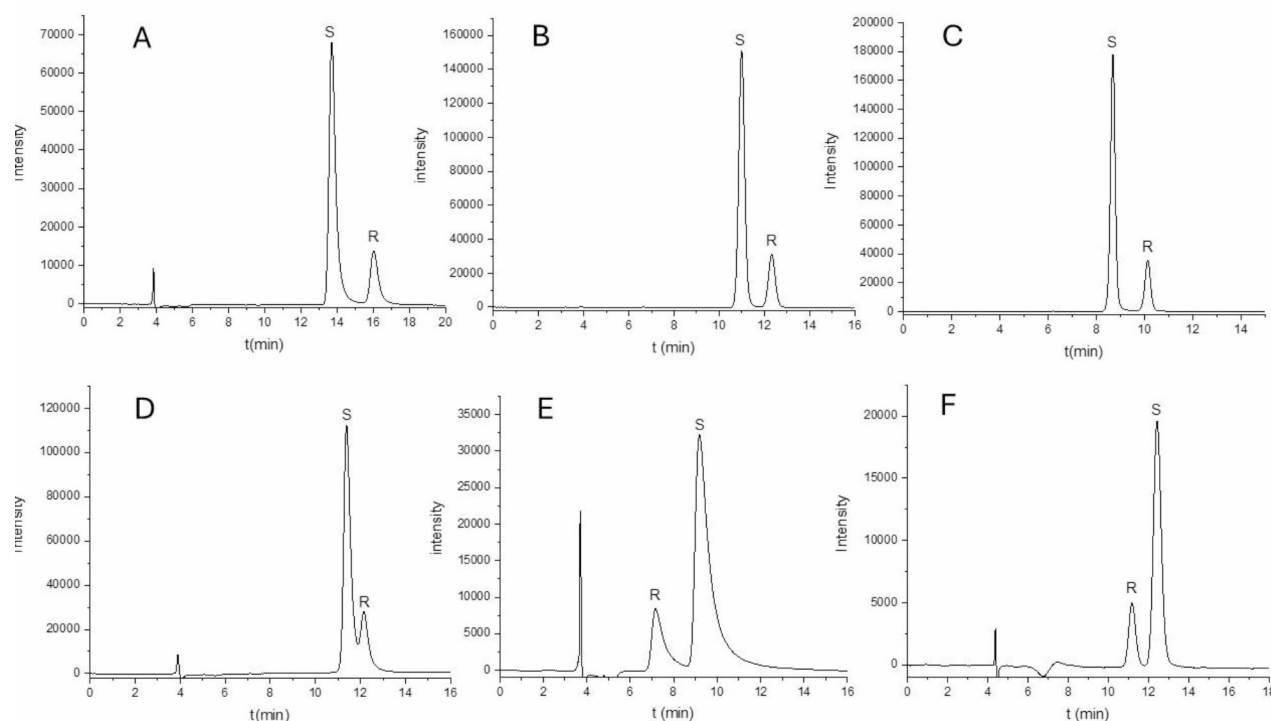


Fig. 3. Experimental chromatograms from preliminary runs. (A) Lux cellulose-2 with ACN: DEA, 100:0.1, flow rate = 0.5 mL/min, (B) Lux cellulose-2 with MeOH: DEA, 100:0.1, flow rate = 0.5 mL/min, (C) Lux cellulose-4 with MeOH: DEA, 100:0.1, flow rate = 0.5 mL/min, (D) Lux cellulose-4 with ACN: DEA, 100:0.1, flow rate = 0.5 mL/min, (E) Lux amylose-2 with ACN: DEA, 100:0.1, flow rate = 0.5 mL/min, (F) Chiralpak AD with MeOH: IPA: DEA, 30:70:0.1, flow rate = 0.7 mL/min.

could potentially facilitate the quantification of enantiomeric impurities without significant interference with the enantiomer. It can also be seen that IPA worsens the enantioseparation in all cellulose-based columns.

In contrast, both the EEO and the targeted minimum resolution threshold were conveniently achieved with Lux Amylose-2 when operated with ACN (Fig. 3E) and Chiralpak AD with MeOH-IPA mixtures (Fig. 3F).

While further optimization on Lux Amylose-2 with ACN might seem logical, a closer examination of the chromatogram revealed concerning results (Fig. 3E): despite achieving a high-resolution value and the appropriate EEO, peak symmetries were unacceptable. Additionally, observed plate numbers were significantly lower than anticipated, even after reasonable adjustments to the flow rate, column temperature and DEA amount, indicating that further improvement in peak shapes was hardly possible. As a result, we decided to proceed with the selection of the Chiralpak AD column with IPA - MeOH mixtures containing 0.1% DEA for further method development instead.

Design space modeling

After the initial selection of the stationary phase, the subsequent step involved establishing a systematic connection between the identified critical APPs and critical APAs. To unveil these correlations, we utilized the DryLab modeling tool. Like the modeling methodologies employed in achiral developments, we began by selecting a meaningful DoE. Building upon the findings of the previous RA step, we chose to investigate the simultaneous effect of the critical APPs within the model: eluent composition (%B = %IPA in MeOH), temperature (T), and flow rate (F).

Given this scenario, the software necessitated a comprehensive 2×2 full factorial experimental plan with two parameters—%B and T—changed experimentally. Variation in flow rate on an experimental basis was deemed unnecessary, as the applied theories of the tool account for this parameter's variability. Following the modeling software's recommendations, when selecting appropriate ranges for the four model input experiments, it was preferable for the two temperature values to differ by at least 20–30 °C. Considering that the manufacturer's recommendation for a polysaccharide column typically sets the maximum operational temperature at 40 °C, choosing 10 °C and 40 °C as input values seemed reasonable. However, selecting the two inputs for %B presented a more compelling challenge. While the proprietary modeling functions have been proven effective in reliably modeling more complex separation cases, in this instance, it was imperative to identify an input range that would yield reproducible results and an optimal modeling fit, ensuring that there was no critical change in chiral recognition. Similar best practices and modeling principles have also been described by Fekete for large molecule applications⁴⁷.

As previously mentioned for amylose-based CSPs, such as Chiralpak AD, enantioselectivity is influenced by both the choice of eluent and the ratio of IPA to MeOH. Therefore, it was necessary to conduct a preliminary

screening to assess how the percentage of IPA in MeOH affects enantioselectivity, selectivity, and retention. In this screening, the percentage of IPA was incrementally increased by 10% in MeOH, ranging from 0% IPA to 100% IPA. All mobile phases contained 0.1% DEA. The results are illustrated in Supplementary Fig. 1. It is evident that enantioseparation can only be achieved in specific eluent mixtures, and notably, the elution order of enantiomers also changes after reaching 30 v/v% of methanol in 2-propanol. However, it is observed that between 40 and 70 v/v% MeOH in IPA, enantioselectivity remains relatively consistent, indicating that this range is well-suited for subsequent modeling. At the same time, it is important to emphasize that selecting inputs outside this range may result in significant changes in separation mechanisms. In such cases, modeling algorithms may only provide results with limited accuracy. Based on these considerations, we selected the following conditions as inputs:

1. Chiralpak AD column, 40 °C, IPA: MeOH: DEA 30:70:0.1 (v/v/v), 0.7 mL/min.
2. Chiralpak AD column, 10 °C, IPA: MeOH: DEA 60:40:0.1 (v/v/v), 0.7 mL/min.
3. Chiralpak AD column, 40 °C, IPA: MeOH: DEA 60:40:0.1 (v/v/v), 0.7 mL/min.
4. Chiralpak AD column, 10 °C, IPA: MeOH: DEA 30:70:0.1 (v/v/v), 0.7 mL/min.

The four acquired chromatograms are depicted in Fig. 4.

Based on the input experiments, DryLab computed a 2-dimensional (%B-T) heat map, showcasing by default the changes in separation (R_s) across the calculated modeling range. This contextualized information can be utilized to delineate the region(s) where the ATP is fulfilled (Fig. 5A). It is evident that around 30%B and 10–15 °C, the required enantioseparation can be attained. As the initial objective of the method was also rapid analysis (<20 min), this heat map was expanded to a third dimension, incorporating flow rate within the range of 0.50–1.00 mL/min. The resultant 3D model is illustrated in Fig. 5B. Here, it's important to emphasize that according to chromatographic fundamentals, modifying the flow rate or column dimensions generally doesn't influence selectivity in isocratic elution. However, such adjustments may affect kinetic performance of the column, as well as changes in backpressure.

Flexible modeling options also allowed us to compute, visualize and study all combination of method parameters fulfilling the selected single or multiple method attributes—within the red displayed area the required analytical targets will be met. Obviously, when considering all critical APAs captured within the ATP, a more restricted space, termed the Method Operational Design Region (MODR), could be derived, illustrating all parameter combinations where both $R_s > 2.0$ and the analysis time is under 15 min (Fig. 5C).

In our study, the final modeling step involved validating the MODR. We meticulously chose nine work points (Supplementary Table 2) within the model and conducted measurements to compare the real retention time (measured data) with the virtual retention time (modeling results), as depicted in Supplementary Fig. 2. While

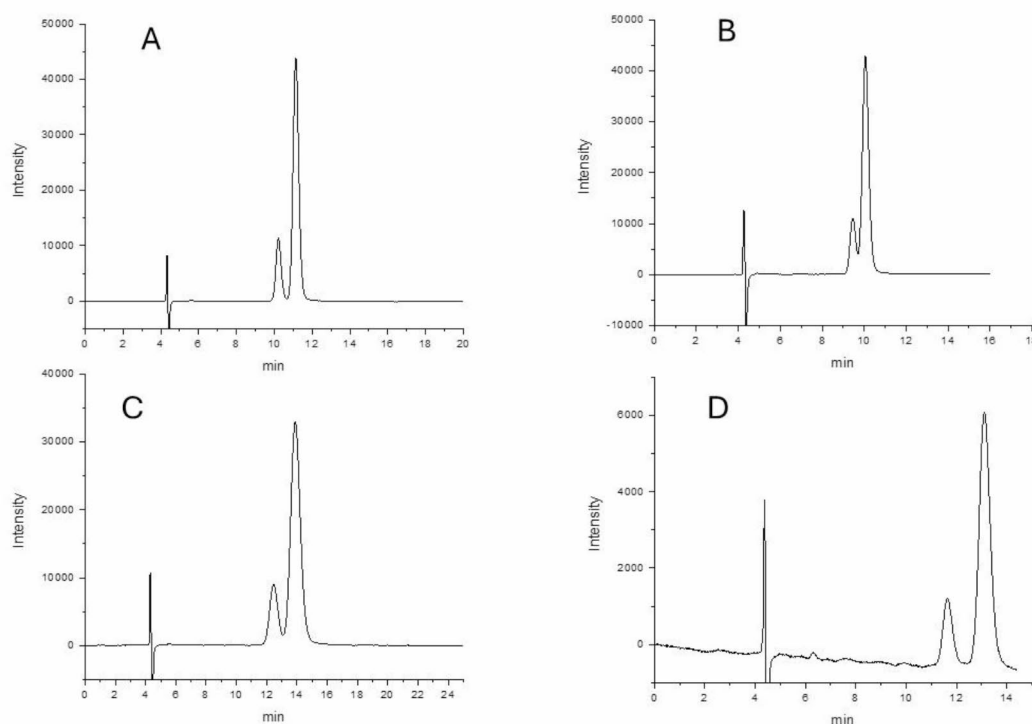


Fig. 4. The chromatograms obtained in case of input conditions (A: 30% IP and 40 °C, B: 60% IP and 40 °C, C: 60% IP and 10 °C, D: 30% IP and 10 °C).

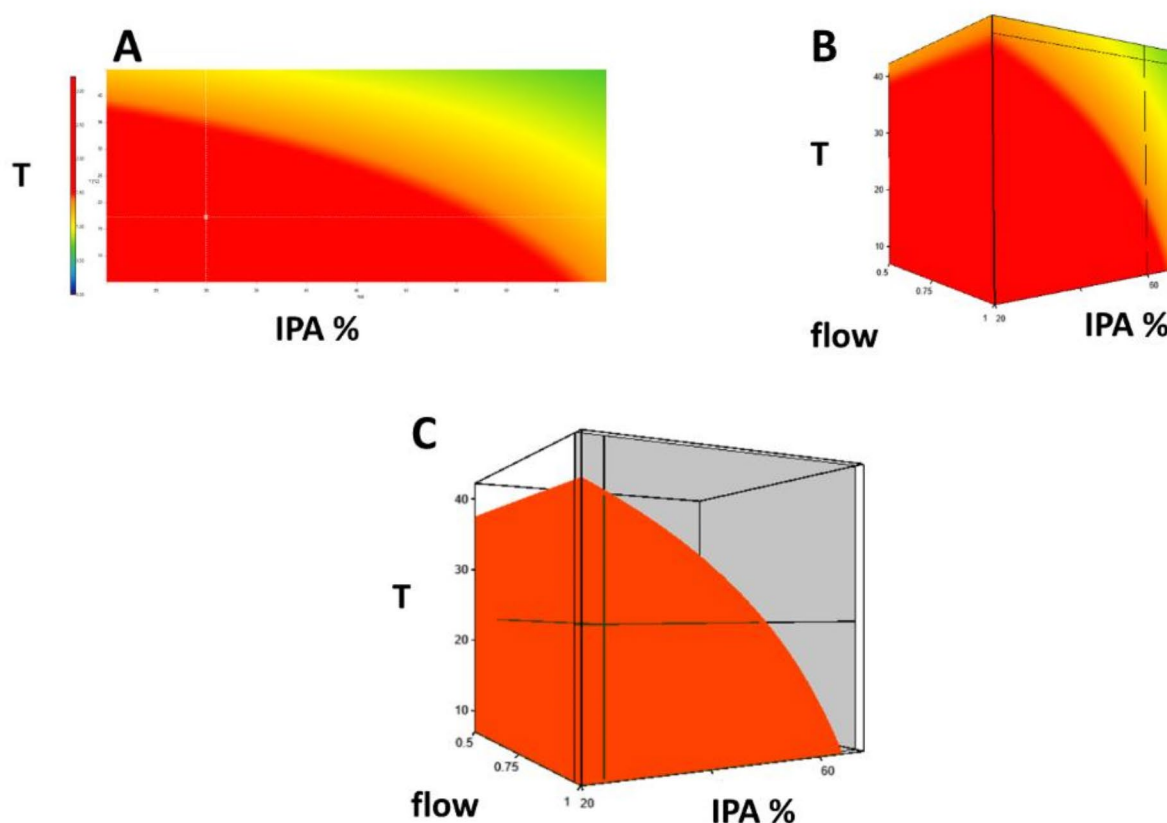


Fig. 5. Modeled IPA%-T heat map depicting separation changes between the two enantiomers on the Chiralpak AD column (A). This visualized map was extended with flow rate in the range of 0.5–1.0 mL/min as third, calculated dimension (B). Method operational design region with $R_s > 2.0$ and analysis time is under 15 min (C).

the modeling results showed a general good agreement with reality, there was slightly more deviation compared to typical accuracy results reported by other authors⁴⁸.

The high R^2 coefficient value (0.99) obtained from the total MODR-verification indicated excellent fitting of the regression model across the range of selected experimental points. Run times were tightly clustered around the fitted linear curve, with a root mean square error (RMSE)—representing the average differences between observed and predicted values—of approximately 0.5 min. The calculated robust estimator (r_r^2) value of 0.86 indicated that the linear correlative model was reliable at explaining the data, even when considering potential irregularities. The slightly lower r_r^2 value compared to the R^2 suggested that the fitted linear model was generally robust and the presence of minor outliers (representing less precise fits between modeled and observed data) had only a limited impact on the overall goodness of fit.

These verification values, especially in consideration of the added flow rate experimental points, underscore the modeling software's suitability to reliably predict the enantioseparation within the previously selected modeling range in PO mode using various MeOH-IPA mixtures (as shown earlier in Fig. 3).

Method validation

The final stage of our development process involved evaluating the performance of a chosen working point for the Chiralpak AD column. We aimed to ensure that the developed analytical procedure meets the related performance criteria objectives outlined in ICH Q2(R2).

Building on the validated MODR, we first selected our final working condition: IPA: MeOH: DEA 30:70:0.1 (v/v/v) with a flow rate of 0.8 mL/min and a column temperature of 10 °C. Figure 6 displays the chromatogram obtained for ozanimod, containing 0.1% chiral impurity, using these optimized parameters.

Furthermore, through the integration of AQbD principles, we enhanced the rationalization of development processes by leveraging information from the previous modeling dataset and generated model. With the utilization of flexible modeling options, we assessed the prospective method performance under the selected working conditions. The experiment-free model robustness option considered all likely deviations for each chromatographic parameter, considering their specified tolerance levels. It then conducted a full-factorial analysis around the chosen point to assess the impact of these deviations on the overall performance of the method.

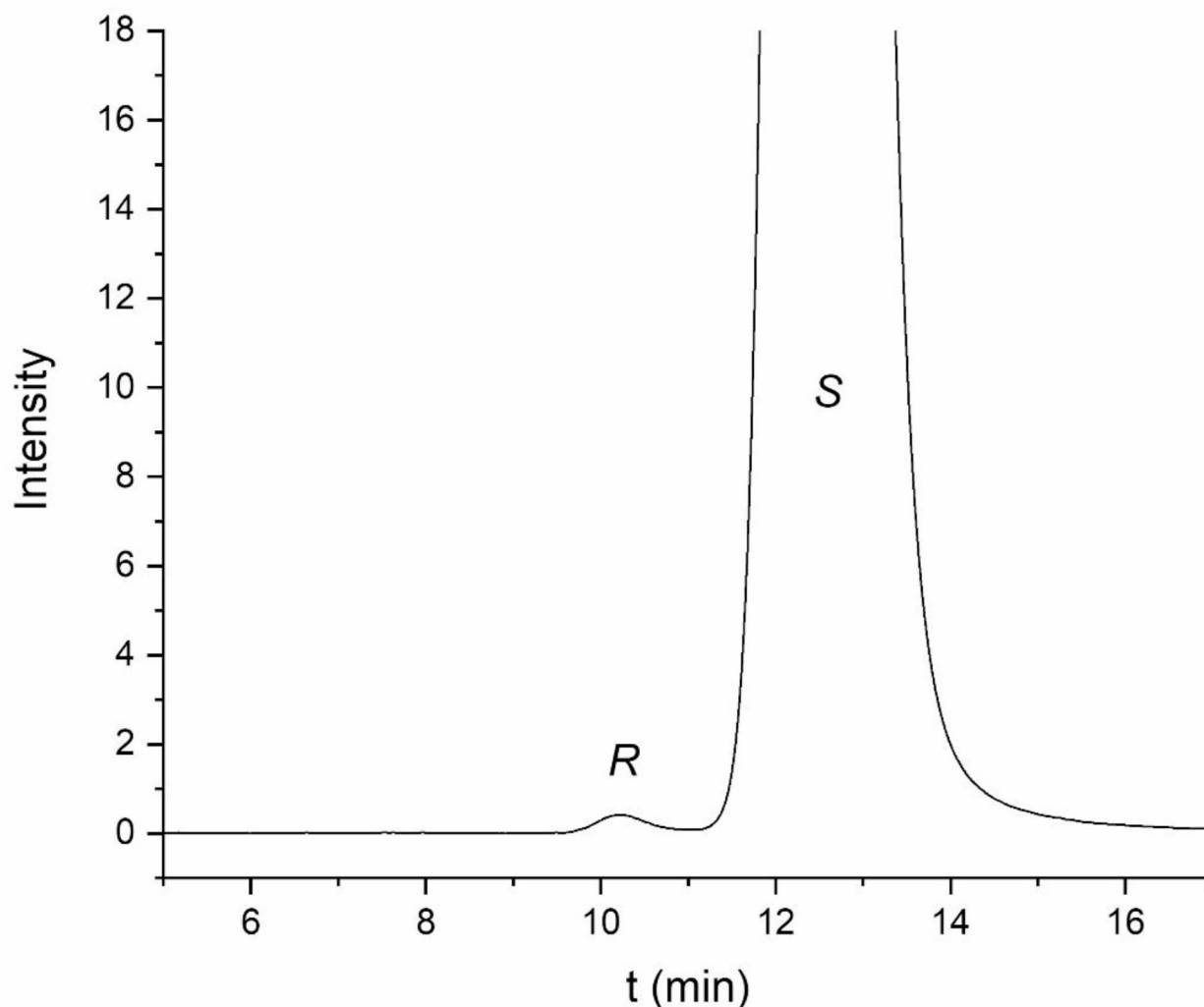


Fig. 6. Chromatogram obtained in optimal working conditions using a Chiralpak AD column with IPA: MeOH: DEA 30:70:0.1 (v/v/v) eluent composition, at a flow rate of 0.8 ml/min, and the column temperature of 10 °C.

Specific values for in silico testing of the selected working condition and the defined tolerances are outlined in Supplementary Table 3. The software calculated results indicated a 100% success rate, meaning that all the virtual combinations ($3^3 = 27$ cases) passed $R_s > 2$, and $t_R < 15$ min.

Once the modeling tool affirmed robust separation was achieved, subsequent validation steps aimed to determine precision, accuracy linearity, limits of detection (LOD), and limit of quantification (LOQ) for the determination of *R*-ozanimod in ozanimod samples was investigated.

The limit of detection (LOD) and limit of quantification (LOQ) of *R*-ozanimod in 1 mg/mL ozanimod samples were established at concentration levels where signal-to-noise ratios of 3:1 and 10:1 were achieved, respectively. The LOD was 0.27 µg/mL, while the LOQ was 0.90 µg/mL for *R*-ozanimod, respectively. The linearity range was investigated in the range of 0.1–0.3% regarding a target concentration of 1 mg/mL ozanimod.

The equation $y = 1.1542x + 0.1224$ with $R^2 = 0.9991$ using 7 different points was derived to represent the relationship between concentration in µg/ml and peak area. The 95% confidence intervals of the y-intercepts included zero, and the residuals exhibited random distributions. Accuracy and precision were evaluated through intraday and interday (intermediate precision) assessments, which involved performing five replicate injections at three different concentration levels (0.1%, 0.2%, and 0.3%). Injections were performed on the same day and repeated over two consecutive days.

The accuracy for *R*-ozanimod, expressed as average recovery percentages, ranged from 99.08 to 101.04%. Intraday precision, represented by RSD percentages, fell within the range of 0.09–1.56%, while the RSD for intermediate precision was below 1.79%. The validation results are summarized in Supplementary Table 4. These validation results confirm that the method is sensitive, linear, accurate, and precise for determining the chiral impurity in an ozanimod sample.

Analytical procedure parameters (APP)	Optimal value	Proven acceptable range (PAR)	Classification
Stationary phase type	Chiralpak AD, 150 × 4.6 mm, 10 µm	–	EC
Flow rate (ml/min.)	0.8	± 0.2	Non-EC
Column temperature (°C)	10	± 5	Non-EC
Mobile phase composition (% IPA)	30	± 5	Non-EC

Table 2. Classification of most relevant APPs in accordance with ICH Q14 and established PARs based on in silico robustness testing.

In line with the recommendation of ICH Q14, as part of method lifecycle management the classification of the most relevant APPs was summarized in Table 2. According to ICH Q14 critical APPs with high impact on method performance are marked as established conditions (ECs). Moreover, the proven acceptable ranges (PARs) for other (non-critical or non-EC) APPs calculated with the aid of in silico robustness testing are also summarized in Table 2. The role of PARs is to facilitate routine applicability of the method by providing higher flexibility compared to a traditional rigid work point report.

Generalization of applied methodology—possibilities and limitations

To draw broader conclusions from this study, thorough preliminary data collection and a well-executed risk assessment to identify critical APPs are essential for developing enantioseparations. Polysaccharide-based columns in polar organic mode are widely used in chiral separations, but it should be noted that similar results can also be achieved using other types of chiral columns. Initial method scouting experiments are still needed, as the selected software cannot foretell which stationary phase will be most suitable for a given enantiomeric pair. This step still heavily relies on a trial-and-error approach. Additionally, the nature of mobile phase components, and the studied range of mobile phase composition are also critical when using polysaccharide-type chiral columns in reversed-phase mode due to the different separation mechanisms. Extending the design space can be challenging, particularly with different IPA: MeOH mixtures, as it only works within a specific range that must be determined before modeling. In other aspects of method development, the mechanistic modeling approach can be applied not only for reversed-phase impurity analysis but also for enantiopurity determination. By using DryLab software, we can precisely define the MODR and assess the robustness of the method.

Conclusion

In conclusion, the development of a direct HPLC method for determining the enantiomeric purity of ozanimod showcases the efficacy of employing polysaccharide-based chiral columns under the PO mode. Through rigorous scouting and optimization phases, it was found that the Chiralpak AD column combined with a MeOH: IPA mobile phase gave the most ideal solution to perform baseline separation within 15 min.

In our work, we effectively implemented systematic elements of the Analytical Quality by Design methodology. Following the ICH Q14 and ICH Q2(R2) guideline, we conducted an initial risk assessment and identified critical APPs, linking them to a systematic model design space. Optimization was facilitated using a mechanistic modeling tool, DryLab software. The resulting validated method, aligned with ICH Q2(R2) guidelines, provided a robust means of assessing chiral impurities in ozanimod samples. Additionally, in silico robustness testing provided insights into critical separation parameters and their impact on enantioresolution. This study also highlights the utility of DryLab software, traditionally designed for reversed-phase achiral separations, in assisting chiral method optimization even in PO mode. However, it also revealed its bottlenecks in modeling chiral separations. The software cannot assist choosing the set of chiral stationary phases for initial screening, and the input has to be limited to avoid separation conditions that lead to significant changes to changes in separation mechanism. However, once the appropriate column selection with mobile phase is made, the modeling tool could reliably predict the optimal method conditions with the highest reproducibility based on only a few experimental runs. Our developed workflow could be applied to other chiral method development regardless of the chemical structure.

Data availability

The datasets used in the present study are available from the corresponding author on reasonable request.

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Author contributions

Elek Ferencz: Methodology, Writing—original draft. Zoltán-István Szabó: Methodology, Writing—original draft, Writing—review & editing, Conceptualization. Arnold Zöldhegyi: Formal analysis, Methodology, Writing—original draft, Software. Gergely Dombi: Investigation, Methodology, Visualization. Gergely Molnár: Writing—review & editing, Project administration, Investigation. Máté Dobó: Methodology, Writing—original draft; Erzsébet Varga: Investigation, Methodology. Imre Molnár: Funding acquisition, Visualization, Resources, Conceptualization, Supervision. Gergő Tóth: Conceptualization, Methodology, Writing—original draft, Software.

Declarations

Competing interests

Imre Molnár is the founder, while Arnold Zöldhegyi is employed by Molnár-Institute for Applied Chromatography, the developer of the DryLab software. All other authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Additional information

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Correspondence and requests for materials should be addressed to G.T.

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