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*Short Communication*

# Simultaneous separation and quantification of pomalidomide chiral impurity using reversed phase–ultra-high-performance liquid chromatography method

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## ABSTRACT

**Objectives:** This study develops and validates an RP-UHPLC method for simultaneously separating and quantifying the R(+) and S(-) enantiomers of Pomalidomide, a 2nd generation immunomodulatory drug, in API using an orthogonal experimental design approach.

**Materials & Methods:** Chromatographic conditions involve an Ultisil Cellu-JR column, a flow rate of 0.8 mL/min, an injection volume of 10 µL, and a 15-minute run time at ambient temperature. Peak purity analysis, facilitated by OpenLab LC software, ensures the reliability of separation by evaluating UV spectra similarity within the 170–700 nm range.

**Results:** The method successfully achieves Pomalidomide enantiomeric separation in the isocratic mode, with retention times of 7.507 minutes for the enantiomer-I and 8.943 minutes for the enantiomer-II.

**Conclusion:** The study underscores the need for diverse chromatographic methods to overcome the challenges posed by Pomalidomide's chiral nature. The results provide valuable insights into optimizing chromatographic techniques for the analysis of chiral impurities, contributing to the advancement of pharmaceutical quality control and research.

**Keywords:** Pomalidomide, Enantiomers, Reversed phase–ultra-high-performance liquid chromatography, Chirality; Impurity

## INTRODUCTION

Pomalidomide (PMD) [4-amino-2-(2,6-dioxopiperidin-3-yl)isoindole-1,3-dione] is 2nd generation of uncharged Immunomodulatory drug which is chiral in nature.[1] PMD has a chiral carbon atom in glutarimide ring and exists as a racemic mixture of the R(+) and S(−) enantiomers. Despite having the same chemical composition, bond structure and bond lengths, the R- and S-isomers of PMD are distinct in their spatial arrangement. These mirror-image forms cannot be superimposed on one another due to their unique three-dimensional configurations. Rapid racemisation and chiral instability of thalidomide analogues in human plasma have been previously reported.[2] Solubility of

this chiral compound PMD is another challenge to align with various separation methods.[3] An orthogonal experimental design may be used to maximise the challenges associated with racemic PMD separation, chiral selector selection and factors influencing enantioseparation.[3] Chromatographic conditions for chiral separation and the selection of the mobile and stationary phases are reviewed to optimise the high resolution of purity using many methods. There is a huge limitation of chromatographic methods reported for the chromatographic separation, detection and simultaneous determination of chiral impurities. Given these challenges, more different chromatographic methods need to be established. The present method was established for the simultaneous separation and determination of PMD chiral impurities of active pharmaceutical ingredients (API).[4-10]

## MATERIALS AND METHODS

Separation for PMD enantiomers was conducted using reversed-phase–ultra-high-performance liquid chromatography (RP-UHPLC) Agilent Infinity 1260 (Agilent Technologies Pvt. Ltd., Germany) in an academic setup. The RP-UHPLC was equipped with a dual pump isocratic system and an autosampler with a diode array detector (DAD) integrated with OpenLab LC software. The chromatographic grade regents such as methanol, triethyl amine and acetic acid (Merk.pvt.ltd) were used to establish the method. Standard PMD (API) was received as a gift sample from the Indian Pharmacopeia Commission, India.

### Mobile phase

#### *Solvent*

500 mL Methanol, 50 µL triethylamine and 50 µL acetic acid mixed thoroughly. The above mixer is filtered through a 0.22 µ nylon membrane vacuum filter and 5 min degassed.

#### *Sample solution*

The optimum amount of racemic PMD standard sample dissolved with methanol in a ratio of 0.2 mg/mL for RP-UHPLC injection.

### Chromatographic condition

Chromatographic column: Ultisil Cellu-JR 5µm 4.6 × 250 mm

Flow rate: 0.8 mL/min

Injection volume: 10 µL

Run time: 15 min

Column temperature: Ambient

Auto sampler temperature: Ambient.

Wavelength: *For* PMD*:* $l_{max}$ *220 nm*

Retention time: *For* PMD Enantiomer-I @ 7.507 min

*For* PMD Enantiomer-II @ 8.943 min

## RESULTS

The PMD exhibited a retention time of 7.507 min for the enantiomer-I and 8.943 min for the enantiomer-II, indicating effective separation. Enantiomeric separation of PMD was achieved using the isocratic mode. Despite the enantiomers being well-separated by 8.9 min, the run was extended for an additional 13 min to ensure the thorough elimination of drug traces from the column and to re-establish the system to its initial conditions, as depicted in Figure 1. The chromatogram in Figure 2 provides a comprehensive view of the entire 13-min run, highlighting the peaks corresponding to both enantiomers of PMD.

The parameters listed in Table 1 are common in UHPLC system suitability tests that assess the performance of the chromatographic system. The area under the chromatographic peak is proportional to the amount of the compound present in the sample. The peak height represents the maximum intensity of the chromatographic peak. Enantiomer-I is slightly higher than enantiomer-II, which is relatively in equal proportion. Retention Factor (k) is calculated by dividing the difference between the retention time of the compound and the void time by the void time. Enantiomer-I has a retention factor of 0.61, while enantiomer-II has a slightly higher value of 0.71. These measured compounds strongly interact with the stationary phase. The in-line close value obtained theoretical plate justified the column efficiency. A tailing factor of 1.0 is considered ideal. Both chromatographs (peak-I and peak-II) show some tailing, enantiomer-I having a tailing factor of 1.216 and enantiomer-II having a lower value of 1.114, suggesting a more symmetrical peak. The peak resolution of 3.626 indicates the separation between two adjacent peaks. It is calculated based on the peak widths and the distance between the peak centres. The above method, in-line with the observed parameter, justified the system suitability of the separation method.

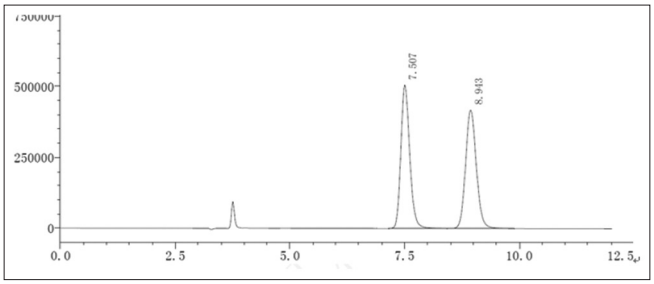


**Figure 1:** Pomalidomide (PMD) chromatograph representing separation of PMD enantiomers. In a chromatogram, the x-axis represents retention time (tR) in minutes while the y-axis shows absorbance intensity measured in milli-Absorbance Units (mAU).

**Table 1:** System suitability depicted from chromatograph of pomalidomide enantioseparation.

| S. No. | Rt | Area | Height | Retention Factor | Theoretical Plates | Tailing Factor | Resolution |
|---|---|---|---|---|---|---|---|
| 1. | 7.507 | 6831452 | 506552 | 0.61 | 7006 | 1.216 | 3.626 |
| 2. | 8.943 | 6820351 | 418021 | 0.71 | 6811 | 1.114 | |

Rt: Retention time

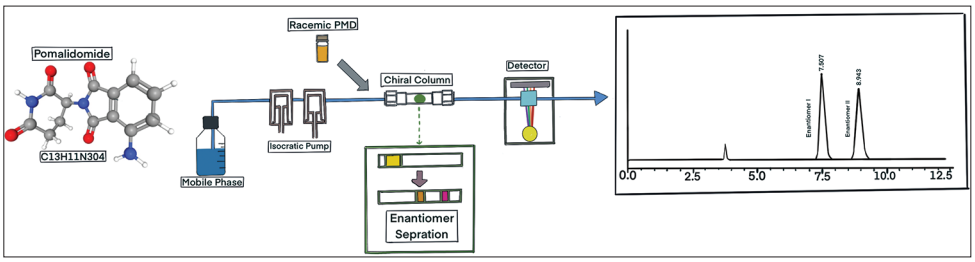


**Figure 2:** Graphical abstract representation pomalidomide separation method.

### Peak purity analysis

Peak purity was determined utilising the OpenLab LC software designed for the Agilent RP-UHPLC system. This assessment was based on the similarity of ultraviolet (UV) spectra within the 170–700 nm range across the peak. The objective of peak purity evaluation was to gather supplementary information to aid in selecting optimal analytical conditions for the specific determination of both enantiomers of PMD. A peak was considered pure if its peak purity index exceeded the single point threshold, resulting in a positive minimum purity index value in Table 1.

## DISCUSSION

This study addresses the intricate challenges associated with the chromatographic separation and analysis of PMD enantiomers, a second-generation uncharged immunomodulatory drug with inherent chirality. The enantioseperation typically relies on thermodynamic processes. This occurs due to varying levels of apparent stability in the short-lived diastereomeric complexes formed by individual enantiomers. In chromatographic techniques, successful separation of enantiomers is only possible when there are substantial thermodynamic distinctions between them.[3] PMD is a molecule with chirality, containing an asymmetric carbon atom. This allows it to exist in two mirror-image forms: The S-isomer and the R-isomer. These isomers are present in equal amounts, forming a 50:50 mixture. They can switch between forms in human plasma through enzyme-mediated and non-enzymatic processes. In the human plasma, the rate at which these isomers are eliminated is slower than the rate at which they interconvert. This means that during a clinically relevant timeframe, the R- and S-isomers can freely transform into each other. As a result, when the racemic mixture (containing both isomers) is administered, there is only a minor difference in the exposure levels of each isomer in the body.[11] It also influenced both its solubility and chromatographic resolution, which posed a significant challenge for enantioseparation in human plasma. The application of an orthogonal experimental design proves instrumental in maximising challenges related to racemic PMD separation, chiral selector selection and factors affecting enantioseparation.

Through an extensive review of chromatographic conditions and the selection of mobile and stationary phases, the study aims to optimise the high-resolution purity determination using multiple methods.[4,5,8,12] Acknowledging the limitations in existing chromatographic techniques for the separation, detection and simultaneous determination of chiral impurities, the study emphasises the necessity for establishing different chromatographic methods.

The developed RP-UHPLC method on an Agilent Infinity 1260 system, equipped with a dual pump isocratic system, autosampler and DAD, successfully separates PMD enantiomers. Chromatographic grade reagents, including methanol, triethylamine and acetic acid, contribute to the method's robustness. The mobile phase, meticulously prepared and degassed, enhances the accuracy of the separation process. Utilising a carefully measured sample solution, the study achieves optimal results with a retention time of 7.507 min for the enantiomer-I and 8.943 minutes for the enantiomer-II.

Peak purity analysis, facilitated by OpenLab LC software, further confirms the effectiveness of the separation by evaluating UV spectra similarity. The extended run time of 13 min ensures the thorough elimination of drug traces, underscoring the method's reliability. System suitability data suggest [Table 1] that the HPLC system is performing reasonably well, as indicated by the retention time, retention factor, tailing factor, peak shape and resolution.[13] The small differences observed between

enantiomer-I and enantiomer-II may be attributed to variations in the enantiomeric separation of PMD.

In line with the present study, a newly developed and validated chiral stationary phase-high-performance liquid chromatography (CSP-HPLC) method for quantifying PMD enantiomers in human plasma. The method employs a Daicel-CSP Chiralpack IA column for optimal enantiomeric separation and achieves efficient separation using methanol and glacial acetic acid mobile phase. Excellent separation of PMD enantiomers with distinct retention times was demonstrated.[12] The method was validated according to ICH(Q2R1) guidelines,[13] showing linearity, sensitivity and specificity, with no interfering peaks observed, indicating high selectivity in complex biological matrices. Overall, the method is presented as a robust and reliable tool for chiral drug analysis in complex biological matrices with potential clinical and research applications.[12]

The established chromatographic method provides a comprehensive solution to the challenges posed by PMD's chiral nature. This research contributes valuable insights into optimising chromatographic techniques for the precise analysis of chiral impurities in pharmaceutical formulations, advancing the field of pharmaceutical quality control and research.

## CONCLUSION

This study provides valuable insights into overcoming challenges associated with PMD chiral resolution, offering a comprehensive approach to optimise chromatographic methods for the analysis of chiral impurities in pharmaceutical formulations. The study findings also highlight the potential importance of enantioseparation techniques in drug development, even for compounds that rapidly interconvert *in vivo*. It suggests that further investigation into single-isomer PMD could provide a viable therapeutic alternative for multiple myeloma.

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