Rapid Separation and Characterization of Chiral Small Molecule Drugs with timsTOF

It is of crucial importance to rapidly analyze chiral molecules throughout the drug discovery and development process to ensure synthesis quality and drug safety [1].

Introduction

Chiral chromatography using HPLC and either a chiral stationary phase, chiral mobile phase additives, or sample derivatization to form diastereometric products can be employed to separate enantiomers [2]. However, selecting and optimizing the right approach can be time consuming and costly. Ion mobility spectrometry – mass spectrometry (IMS-MS) is an analytical technique that separates molecules as gas phase ions based on their interactions with a low-energy neutral collision gas prior to mass spectrometric analysis. This technology has the potential to

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reveal structural diversity of chemical species based on their size and shape and is thus used for rapid analysis of isomeric species by way of measuring their collisional cross section (Å^2, CCS) on a millisecond timescale. In this study, the high resolving power of trapped ion mobility spectrometry (TIMS) is used for the rapid and successful separation of thalidomide, oxazepam, and verapamil enantiomers.

Method

Thalidomide, oxazepam, verapamil and solvents were purchased from Sigma. Stock solutions (1.0 mg/mL) of each analyte were prepared in methanol. Working solutions of 50 µg/mL and 1.0 µg/mL were used for direct infusion and flow-injection analysis respectively, with LC-MS acquisitions performed using an Elute UHPLC and timsTOF Pro 2 with TIMS enabled in ESI positive mode. Data processing was conducted in Data Analysis 5.3 software (Bruker).

A previously established 4D-Metabolomics method with *m/z* ranging from 20–1300 and a mobility range of $1/K_0$ 0.45–1.45 V·s/cm^2 was used and further optimized by adjusting ion mobility resolution mode, accumulation time, duty cycle, ramp time and ion mobility range. A maximum ramp time of 959.9 ms was used to achieve optimal ion mobility separation. Both mass and CCS calibration were performed prior to data acquisition.

Results

Thalidomide racemate separation and characterization

Thalidomide is infamous for causing more than 10,000 children born with a range of severe deformities and thousands of miscarriages in late 1950s and early 1960s. Almost 20 years later, Blaschke et al. [3] discovered that only the S-enantiomer is responsible for causing birth defects, while the R-enantiomer is good for preventing morning sickness in expecting women. Figure 1 shows the structures of the enantiomers of thalidomide, oxazepam, and verapamil.

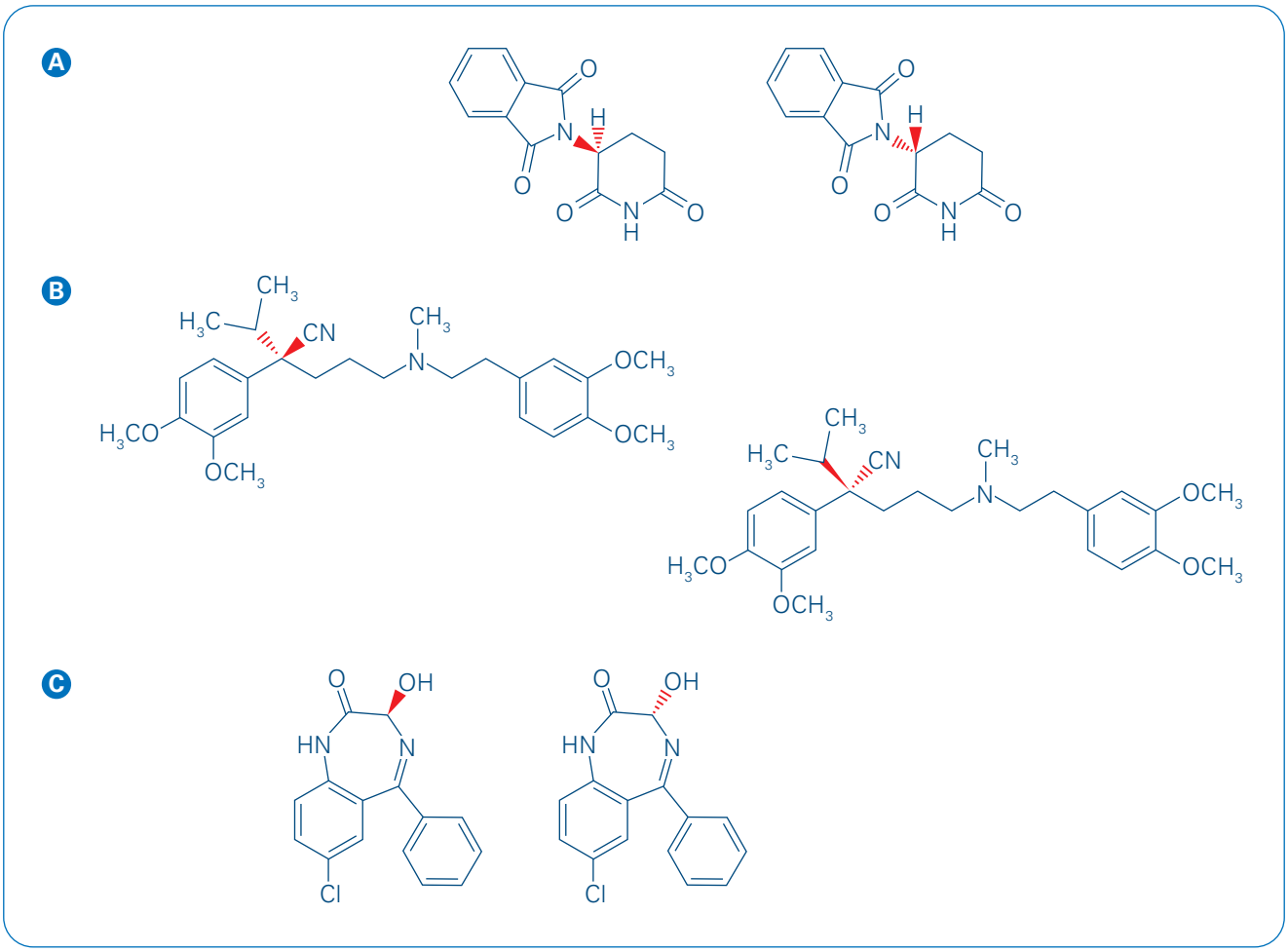

Figure 1: Structures of enantiomers of thalidomide (A), verapamil (B), and oxazepam (C). Left side is R, right side is S. Chiral centers are highlighted in red.

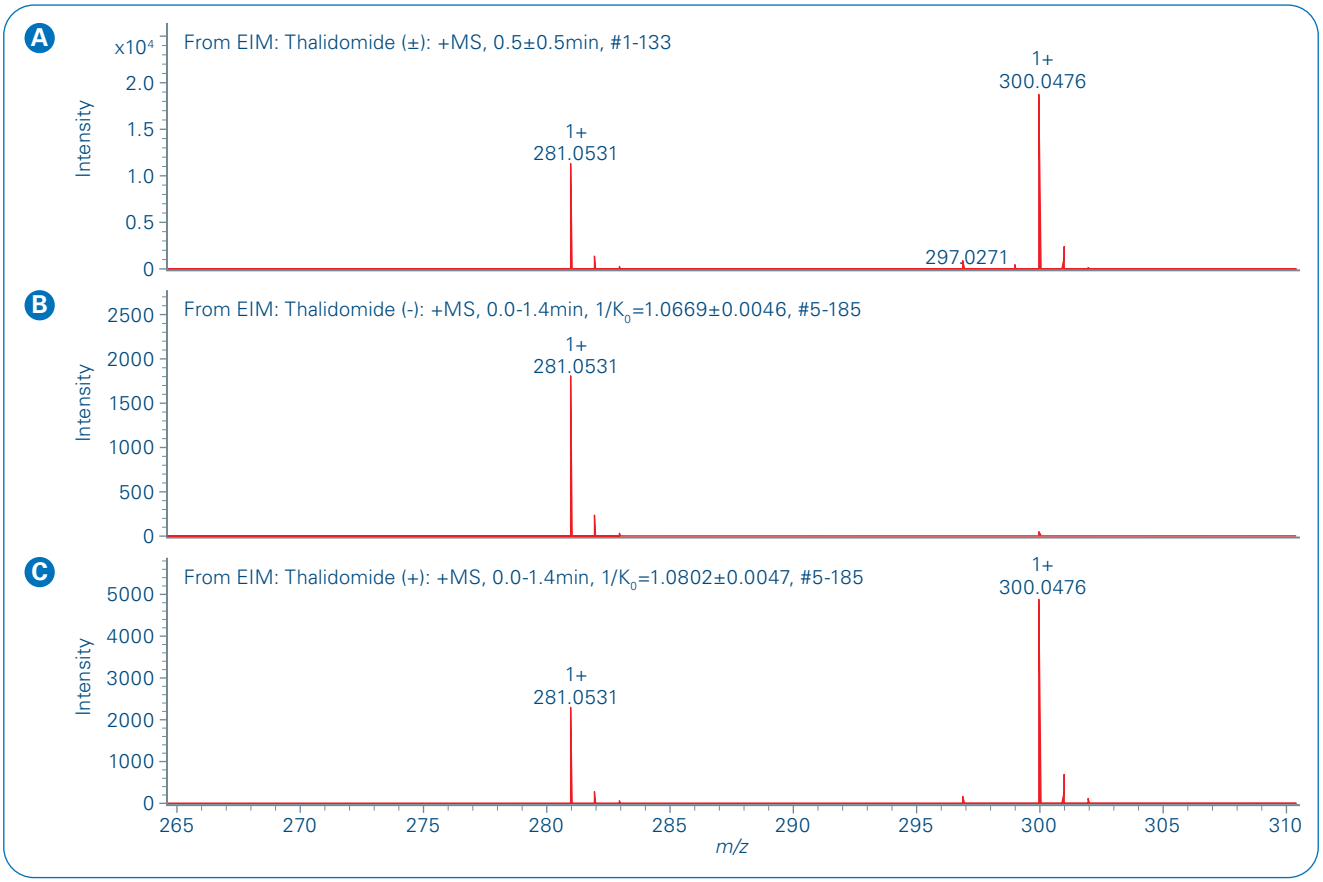

Figure 2: Mass spectra of $[M+Na]^+$ for (R,S)-thalidomide (A), (S,-)-thalidomide (B) and (R,+)-thalidomide at 281.0531 m/z.

Thalidomide mass spectrum, Ion Mobility, and CCS

Pure enantiomers of (R,+), (S,-), and racemate (R,S) of thalidomide were introduced into a timsTOF Pro 2 by direct infusion using a syringe pump, also by flow injection analysis LCMS. Its major sodium adduct ion $[M+Na]^+$ of 281.0532 *m/z* was observed as well as the $[M+Na]^+$ peak of hydrolysis product 2-phthalimidoglutaric acid at 300.0476 *m/z*. The full MS spectra from the extracted ion chromatogram (EIC) and extracted ion mobilogram (EIM) of thalidomide are shown in Figure 2 and Figure 3. The experimental CCS values observed are within 4% of the predicted dimer value of 236 Å².

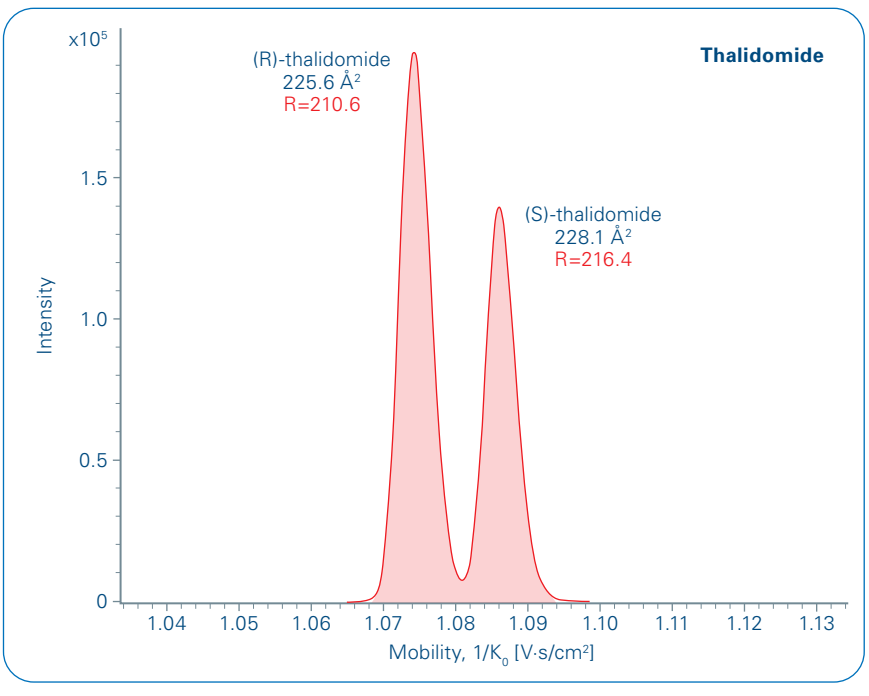

Figure 3: EIM of racemate of thalidomide using the $[M+Na]^+$ ion species.

The drift time profiles of (R,+) and (S,-) thalidomide reported by Philips et al. [4] are identical and have more than one peak in both positive and negative ion modes, and thus may have racemized in solution and could not be fully separated. We speculate that drift tube IMS lacked the required resolving power to effectively separate such minor structural differences.

When analyzed using the timsTOF Pro 2, both R and S enantiomers of thalidomide are resolved with $R_s \geq 1.4$, and each shows only one peak with excellent symmetry. By spiking and testing different ratios of (R)-thalidomide and (S)-thalidomide, it was identified the first EIM peak belongs to (R)-thalidomide. Since TIMS utilizes two sequential analyzer cells, one for ion accumulation and the second to release ions by reducing the electric field potential, the technology offers substantial ion mobility resolving power (resolution R~200) by design and make chiral separation possible based on analyte's shape and size.

Verapamil and oxazepam ion mobility separation

Similarly, racemic mixtures of oxazepam and verapamil were analyzed using the timsTOF Pro 2. With optimized experimental conditions, full baseline ion mobility separation of R and S verapamil enantiomers (*m/z* = 455.2904 $[M+H]^+$) was achieved. This result is consistent with the recently proposed mechanism of enantiomer separation by protonation-induced chirality [5]. For oxazepam (*m/z* = 287.0582 $[M+H]^+$), approximately 80% ion mobility baseline separation was achieved. Both results are illustrated in Figures 4 and 5 below.

The ion mobility resolution and CCS of each compound is summarized in Table 1 and compared to previously reported data [6,7].

The flow-injection analysis approach for IMS-MS of chiral drugs was also performed, achieving the same resolution and CCS values with much faster sample throughput. This highlights TIMS as an effective method for screening chiral drugs. As ultra-high resolution mode is typically applied to achieve high ion mobility resolution to differentiate enantiomers, both mass calibration (TOF) and ion mobility (TIMS) calibration prior to chiral drug

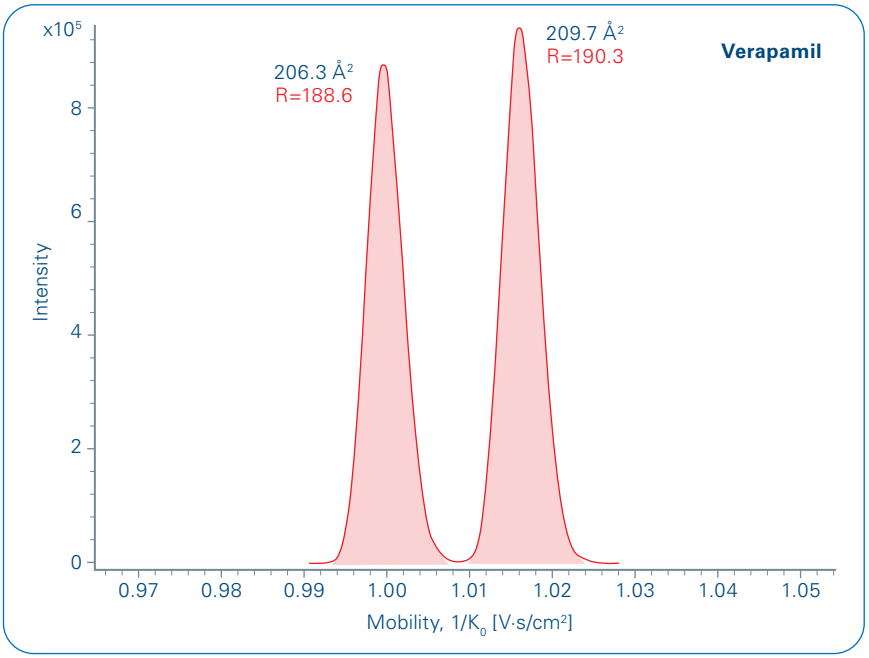

Figure 4: EIM of racemate of verapamil using the $[M+H]^+$ ion species.

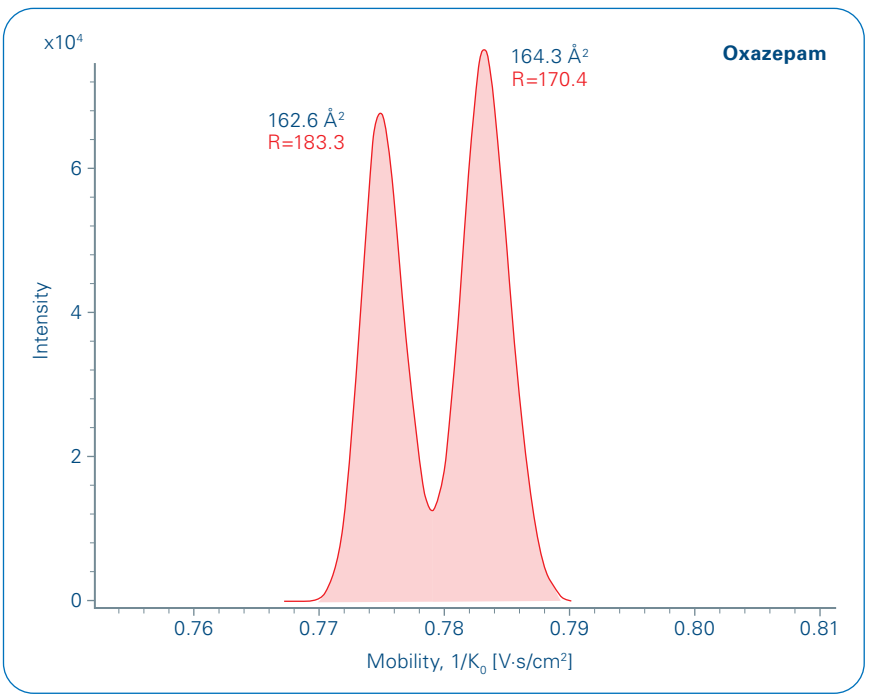

Figure 5: EIM of racemate of oxazepam using the $[M+H]^+$ ion species.

separation are required to ensure the timsTOF is achieving accurate and reliable data quality. With the optimized ion mobility separation conditions, this method can be applied to LCMS experiments to quantify enantiomers. However, this type of workflow is normally used for targeted chiral separations. When performing untargeted ion mobility separations of chiral compounds, a survey scan is required to assess and apply optimized TIMS experimental parameters for further mass analysis. This is achieved by enabling Parallel Accumulation Serial Fragmentation (PASEF). The timsTOF Pro 2 is capable of acquiring and analyzing a single TIMS-MS scan to determine optimal resolution prior to acquiring fragmentation data. This is a fast and cost-effective method for untargeted analysis of separable enantiomers.

In summary, the Bruker timsTOF Pro 2 was able to achieve an average ion mobility separation resolution of 198 for this particular study, and capable and resolving chiral drugs without the need for chiral chromatography or any other chiral selector mechanism. The CCS of enantiomers measured agreed well with reference values and clearly demonstrates the ability to differentiate chiral drug molecules as a 4th dimensional parameter.

Table 1: Ion mobility resolution and CCS of thalidomide, verapamil, and oxazepam.

Name	EIM (*m/z*)	Enantiomers	Mobility, $1/K_0$	IM Resolution	CCS [Å^2] (measured)	CCS [Å^2] (reported)
Thalidomide†	281.0533±0.1	R	1.074	225.6	210.6	N/A
		S	1.086	228.1	216.4	
Verapamil‡	455.2904±0.1	I	1.000	188.6	206.3	210.2*
		II	1.016	190.3	209.7	
Oxazepam‡	287.0582±0.1	I	0.775	183.3	162.6	162.3*
		II	0.783	170.4	164.3	

*† Observed [M+Na]$^+$; ‡ Observed [M+H]$^+$; * Reported CCS values from PubChem.*

Conclusion

- Drug enantiomers were successfully separated using direct infusion and flow injection analysis methods using Bruker timsTOF Pro 2 (IM resolution ~200) without any chiral selectors.
- CCS values of enantiomers can be used to differentiate and characterize chiral drugs and drug-like molecules as a 4th dimensional parameter.
- Trapped ion mobility MS has increased resolving power and is becoming a powerful analytical technique to analyze (separate, differentiate, and characterize) more conformational isomers.

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