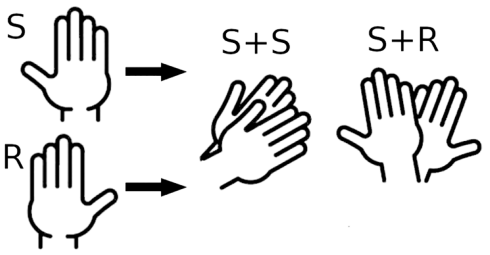
Quantitation of Enantiomeric Excess in an Achiral Environment Using Trapped Ion Mobility Mass Spectrometry

Joseph Czekner,* Erik K. Schneider, Patrick Weis, and Manfred M. Kappes*

ABSTRACT: We present a novel, straightforward method to determine the enantiomeric excess (ee) of tryptophan (Trp) and *N-tert*-butyloxycarbonyl-*O*-benzylserine (BBS) solutions without chiral additives. For this, lithium carbonate, sodium carbonate, or silver acetate was added to solutions of Trp or BBS. Singly negatively charged dimer and trimer clusters were then formed by electrospray ionization and analyzed using trapped ion mobility spectrometry (TIMS) and time-of-flight mass spectrometry. When a solution contains both enantiomers, homo- and heterochiral clusters are generated which can be separated in the TIMS-tunnel based on their different mobilities using a nitrogen buffer gas. The ratio of homochiral to heterochiral clusters shows a binomial distribution and can be calibrated with solutions of known ee to yield ee measurements of samples with better than 1% accuracy. Samples can be prepared rapidly, and measurements are completed in less than 5 min. Current instrumental limitations restrict this method to rigid molecules with large functional groups adjacent to the chiral centers. Nevertheless, we expect this method to be applicable to many pharmaceuticals and provide the example of 1-methyltryptophan to demonstrate this.



INTRODUCTION

Obtaining enantiopure products is often a goal in pharmaceutical synthesis. For example, D-ethambutol is used in the treatment of tuberculosis while L-ethambutol can cause blindness in patients.[1,2] Therefore, it is necessary to develop simple and quick methods to determine enantiomeric excess (ee) in order to protect patients and accurately quantitate any adverse effects. Mass spectrometry (MS) can be an ideal technique because it does not require a large amounts of samples and can achieve high accuracy.[3] However, MS alone is a "chiral-blind" technique because enantiomers respond identically to the applied electromagnetic fields. However, modifications can be made to elicit different responses from enantiomers.

One such modification is known as the kinetic method (KM).[4,5] KM measures different fragmentation patterns between clusters of hetero- or homochiral enantiomers. It requires sufficient binding energy differences and simultaneous monitoring of multiple fragmentation channels and is therefore often hard to apply. Another method is the chiral recognition ratio (CR) method.[6] This method is similar to KM except that it uses aggregates of the enantiomers to be studied with a chiral reference compound. The advantage of this method is that the reference compound can be selected so as to optimize the differences in the fragmentation pattern—ideally in a single fragmentation channel. In addition to collisional fragmentation, the CR method has also been implemented for UV photodissociation.[7,8]

Ion mobility (IM) can be added to MS for an additional dimension of separation. IM separates ions based on their "shape" by using the opposing forces imparted by collisions with inert neutral buffer gases and electric fields.[9] This comes in two flavors for chiral separations. The first involves adding a pure enantiomer of an unreactive, neutral chiral molecule to the buffer gas. This interacts more strongly with one of the two enantiomeric ions (typically volatilized from solution samples by electrospray ionization (ESI)) resulting in separation.[10] Then, relative MS peak intensities can be integrated to quantitate ee in the solution being probed. There are only a few inexpensive enantiopure neutral chiral molecules with sufficiently high vapor pressure. Furthermore, this method is difficult to model. Instead a second chiral selective IM-MS technique, similar to the CR method, is more commonly used.[11−15] Here, an enantiopure chiral reference compound complexes differently with each of the two enantiomeric ions of interest to yield charged aggregates with different shapes. These can then be separated using IM (to yield a so-called mobilogram) before analysis with MS to determine % ee of the sample. Related to this, covalently bound diastereomers of amino acids can also be preformed in condensed phase and then separated using trapped ion mobility spectrometry (TIMS).[16,17]

The common theme of the aforementioned separation techniques is that they always rely on chiral modifications to the environment or even to the chiral ions themselves. Here, we demonstrate that chiral separation can also be achieved in an achiral environment by formation and IM analysis of clusters comprising the enantiomeric ions of interest together with achiral counterions. In other words, in the presence of such achiral counterions, a molecule can be used as its own chiral reference compound and homochiral clusters of the same enantiomer can be separated from heterochiral clusters consisting of both enantiomers using TIMS with a nitrogen buffer gas. The ratio of hetero- to homochiral clusters can then be obtained directly from the mobilograms to accurately determine the ee of the sample solution. We demonstrate this using two test cases, tryptophan (Trp) and N-*tert*-butyloxycarbonyl-*O*-benzylserine (BBS). We also show an application of the method to 1-methyltryptophan (1-Met-Trp), a potential pharmaceutical compound, and then end with a brief discussion of present limitations and future perspectives of this approach.

METHODS

Singly negatively charged clusters were generated and probed by ESI coupled with a Bruker timsTOF Pro trapped ion mobility mass spectrometer. Additional experimental details are given in the Supporting Information (SI).

Sample Preparation. Stock solutions of the pure enantiomers were prepared in DI water with the following concentrations: 20 mM for L- and D-Trp, 0.5 mM for L- and D-BBS, and 5 mM for L- and D-1-Met-Trp and stored at 4 °C. Stock solutions of the carbonate salts were prepared in DI water with approximate concentrations of 5−10 mM and stored at room temperature.

Various enantiomeric excess (ee) solutions were prepared fresh by pipetting the appropriate volume of each enantiomer stock solution. We tested two common ESI solvent mixtures, 1:1 H_2O/MeOH and 1:4 H_2O/ACN, and saw no difference in behavior. Carbonate salt or silver acetate and ee solutions were combined in equivolume amounts and diluted by 10 times in the ESI solvent mixtures. An appropriate amount of the solvents were added to keep the ratios stated above. We concentrated on dimeric and trimeric aggregates held together by metal cation bridges which are the dominant cluster anions formed.

Overview of Trapped Ion Mobility Mass Spectrometry. All measurements were performed on a Bruker timsTOF Pro. Conditions were optimized each day. This led to small day-to-day fluctuations of CCS maxima in different measurements of the same sample as indicated, e.g., in Figure 1. The instrument was always operated in negative mode since only anions were studied.

TIMS separates ions based on their "shape" by using opposing forces of an electric field gradient (EFG) and buffer gas flow (nitrogen in our case). TIMS measurements are carried out using a so-called "TIMS-tunnel" in which electrostatically trapped ions are sorted in space according to their differing mobilities. Ions with higher CCS will penetrate further into the trap, and the EFG can be lowered in steps to control when analytes elute from the TIMS tunnel. Details concerning the operating principle can be found in ref 18. Specific settings used here are provided in section 2b of the SI. TIMS does not operate on first-principles so it must be calibrated for each setting, which we did daily. We used Agilent

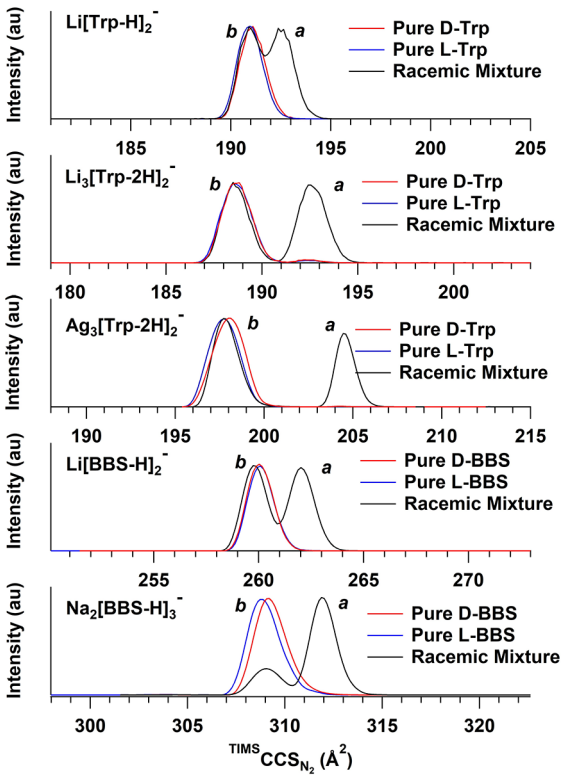


Figure 1. Comparison of test cases with tryptophan and BBS for pure solutions and racemic mixtures. In each mobilogram, *a* denotes heterochiral clusters and *b* denotes homochiral clusters.

Tune-Mix as purchased and the drift tube CCS values ($^{DT}CCS_{N2}$) that were reported previously.[19] TIMS yields inverse mobilities ($1/K_0$) that can be converted to CCSs ($^{TIMS}CCS_{N2}$) via the Mason−Schamp equation (S1).[20]

$$CCS = \left(\frac{3}{16N}\right)\left(\frac{2\pi}{kT}\right)^{0.5}\frac{q}{\sqrt{\mu}K_0} \quad (1)$$

Here, q is the charge of the ion, N is the number density of the collision gas, μ is the reduced mass of the ion and N_2 buffer gas, k is the Boltzmann constant, and T is the temperature in Kelvin.

RESULTS AND DISCUSSION

Mobilograms of Trp and BBS. The mobilograms shown in Figure 1 document the resolution of homo- versus heterochiral forms of metal-bridged Trp dimer anions, a lithiated BBS dimer, and a sodiated BBS trimer anion. The nomenclature used to label the ions, e.g., $Li[Trp\text{-}H]_2^-$, indicates the number of singly deprotonated Trp (or BBS) units bridged by a given number of M^+ counterions. The corresponding measured collisional cross sections (CCSs) are provided in Table 1.

In Figure 1 the homochiral clusters (i.e., L−L and D−D or L−L−L, and D−D−D), respectively, have identical CCSs and are denoted as peak *b*. The small fluctuations of CCS maxima in different measurements correspond to day-to-day fluctuations of the timsTOF spectrometer.[21,22] The heterochiral clusters, peak *a*, are diastereomers of the homochiral clusters, form different structures, and can be separated from them by timsTOF. For example, in the case of $Li_3[Trp\text{-}2H]_2^-$, L−L and D−D have identical CCSs, whereas L−D/D−L are a little larger.

Table 1. Summary of All Measured CCSs for the Homo- and Heterochiral Clusters

Cluster	*m/z* (amu)	Homochiral CCS (Å^2)[a]	Heterochiral CCS (Å^2)
$Li[Trp\text{-}H]_2^-$	413.18	191.1 ± 0.3	192.8 ± 0.3
		191.5 ± 0.3	
$Li_3[Trp\text{-}2H]_2^-$	425.20	188.2 ± 0.3	192.4 ± 0.2
		188.7 ± 0.2	
$Ag_3[Trp\text{-}2H]_2^-$	726.85	198.0 ± 0.4	204.6 ± 0.4
		197.7 ± 0.6	
$Li[BBS\text{-}H]_2^-$	595.28	260.7 ± 1.4	262.1 ± 0.6
		260.0 ± 0.6	
$Na_2[BBS\text{-}H]_3^-$	928.38	309.5 ± 1.1	311.9 ± 0.7
		309.4 ± 0.8	
$Li[1\text{-}Met\text{-}Trp\text{-}H]_2^-$	441.21	197.0 ± 0.6	198.6 ± 1.2
		196.9 ± 0.6	

[a]The first value listed is the pure D enantiomer and the second is the pure L enantiomer.

This accounts for the approximate 1:1 ratio observed. Likewise, for $Na_2[BBS\text{-}H]_3^-$, all six permutations of the diastereomers, i.e., L–L–D, L–D–L, D–L–L, etc., have the same CCSs within experimental resolution, which accounts for the approximate 3:1 intensity ratios of *a*:*b* observed and suggests statistical formation of clusters during ESI.

This is the first example of separating homo- and heterochiral dimers and trimers of the same monomer units using IM-MS and offers a simple way to quantitate the ee of chiral mixtures. It does not rely on any additives to the sample other than a salt. Two peaks are present in the mobilograms if the sample contains both enantiomers. Their ratios allow one to determine ee as will be demonstrated below. Note that dimer signals can be used for this, offering more intense signals and greater sensitivities than when using abundances of larger heteroclusters as described in two related previous reports.[23,24]

Quantitation of ee. The quantitation method will now be briefly described. For this we define x_D and x_L as the mole fractions of each enantiomer in solution (totaling 1). Then, the percent ee of D (or L) can be expressed in the usual manner (eqs 2 and 3).

$$1 = x_D + x_L \tag{2}$$

$$ee_D = (2x_D - 1) \times 100 \tag{3}$$

For this to work as an analysis method the ratios (r) of the areas under peaks *a* and *b* must be expressible in terms of a well-defined function of ee over a wide sample composition range. To check for this we derived a general relationship between r and x_D for dimers and trimers, respectively, as given in eq 4 (see the SI for the derivations). Here, p is a fitted constant that accounts for any nonstatistical effects, such as energetic preference for homo- or heterochiral formation, different ionization efficiencies, different fragmentation energies, systematic error in mole fraction determination, etc. and n is number of monomers in the clusters, e.g., 2 for dimers and 3 for trimers. One can easily substitute between x_D and ee_D (or x_L and ee_L).

$$r = p\frac{(nx_D - nx_D^2)}{(1 - nx_D + nx_D^2)} \tag{4}$$

In Figures 2 and 3 we show corresponding fits for several different calibration samples and ions. In all cases, the sum of errors squared is small. For perfectly statistical conditions, p should be 1. We generally observe values near one which suggests that nonstatistical effects are small especially for the alkali interlinked clusters.

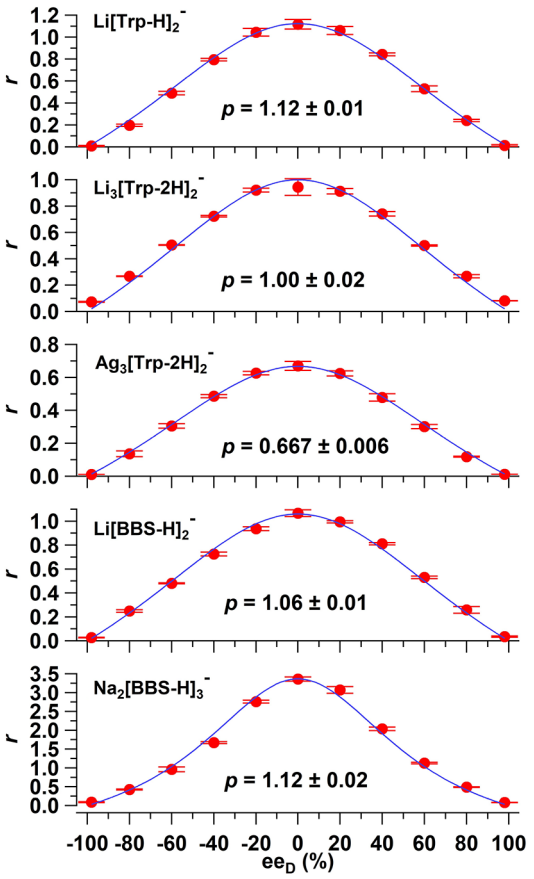


Figure 2. Plots of r versus ee with fitted binomial functions. The ee is calculated relative to D-ee.

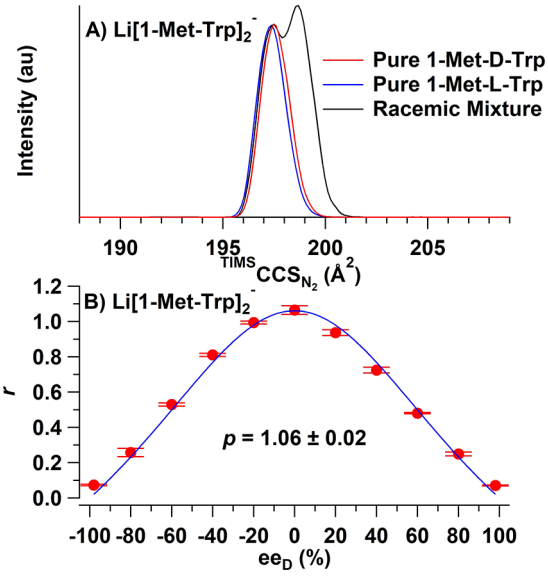


Figure 3. Example of our method applied to 1-Met-Trp: (A) comparison between the pure solutions and racemic mixture; (B) fitted binomial function.

Using the fitted functions, it is also possible to improve estimation of the purities of the enantiopure stock solutions (by treating both purities as independent variables with which to maximize the fit quality for p). Using the $Li_3[Trp\text{-}2H]_2^-$ data set, we obtain purities of 98.8% ± 0.6% for D-Trp and 98.9% ± 0.1% for L-Trp which are each within the manufacture's stated purity. Similar results were obtained using $Ag_3[Trp\text{-}2H]_2^-$. $Li[Trp\text{-}H]_2^-$, $Li[BBS\text{-}H]_2^-$, and $Na_2[BBS]_3^-$ were not resolved well enough to accurately fit the pure samples, and we could not refine the ee using these clusters.

Current Limitations. One caveat to this technique is that fitted *r* vs ee (%) curves are necessarily symmetric. Therefore, a given *r* value is by itself not enough to identify which enantiomer is in excess. For this, a small amount of either of the pure enantiomers can be added to the sample solution to see in which direction ee shifts.

Currently, there are some limitations preventing this from being broadly applicable. For example, we could not resolve mobility differences between alkali doped phenylalanine or tyrosine dimer anion diastereomers. This is presumably because one needs larger functional groups attached to the chiral centers to cause enough steric hindrance to yield resolvably different shapes of homochiral versus heterochiral aggregates. For the instrumental resolution available with timsTOF, this is likely not possible for the smaller amino acids. It is conceivable that this limitation could be overcome by using different counterions, cationic clusters, or even larger aggregates than those studied here. We have not tried all possibilities yet. One can see that using Ag instead of Li in Trp clusters increases the separation. However, we could not resolve the differences in the analogous clusters using Na and K. This means the ionic radii alone is not the sole factor in the separation of these clusters. In addition, Ag induces somewhat larger nonstatistical effects compared to the alkali ions probably reflecting more anisotropic interactions. Another approach could be to improve the IM resolution. Currently cyclic-IM[25] or SLIM[26] (structures for lossless ion manipulation) can achieve 2−4 times higher resolution compared to the timsTOF used in this study.

Application to a Potential Pharmaceutical: 1-Met-Trp. However, we propose that our method could be of interest for rapid screening of the chiral purity of pharmaceutical compounds. A recent review noted that seven of the top 10 selling pharmaceuticals in the United States are single enantiomer compounds, all of which are larger than Trp or BBS and contain many functional groups.[27] Along these lines, we have applied our method to 1-methyltryptophan (1-Met-Trp) enantiomers, of which 1-methyl-D-Trp recently underwent phase 1 trials to treat brain tumors.[28,29] The mobilograms and calibration are shown in Figure 3. The accuracy and fit quality for the 1-Met-Trp data set is similar to the amino acid (derivative) test cases shown in Figure 2, despite the peaks only being separated by 1.6 Å^2. Again, these peaks are not fully resolved so we could not refine the ee of the pure samples.

CONCLUSIONS

In conclusion, we present the first IM-MS based determination of ee without added chiral modifiers. We show that chiral compounds can be used as their own modifiers in the presence of a counterion in an achiral environment given sufficient IM resolution. Sample preparation, calibration and measurement is quick and the method is accurate over the full ee range. Recent improvements in commercial IM-MS technologies (e.g., cyclic-IM or SLIM) suggest future uses for rapid screening of chiral pharmaceutical compounds.

The simplicity of this approach has been recognized independently by the authors of ref 30 who during review of our contribution published a very similar study using an alternative high-resolution IM platform.[30]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.2c00136.

Additional details of the experimental methods and materials used along with derivations of the fitting functions (PDF)

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Author Contributions

J.C. and M.M.K. conceived of the project, and J.C. carried out the measurements and analysis. All authors contributed to discussion and writing of this manuscript.

Notes

The authors declare no competing financial interest.

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