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Metal-ion free chiral analysis of amino acids as small as proline using high-definition differential ion mobility mass spectrometry

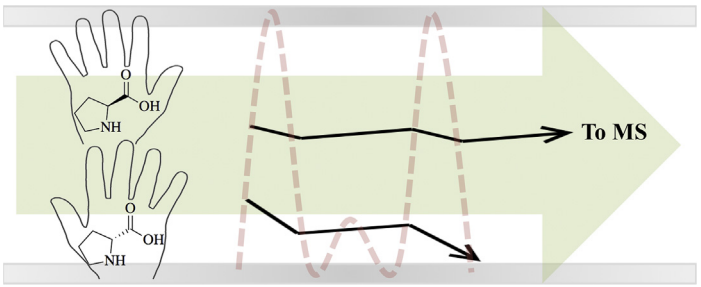
J. Diana Zhang, K.M. Mohibul Kabir, William A. Donald*

School of Chemistry, University of New South Wales, Sydney, NSW, 2052, Australia

HIGHLIGHTS

- A metal-ion free DMS-MS method was developed for the rapid chiral analysis of small amino acids.
- L- and D-enantiomers of cysteine and proline were separated using diastereomeric proton-bound dimers.
- Enantiomeric excess of molecules as small as proline can be quantified in a separation process that takes milliseconds.

GRAPHICAL ABSTRACT



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ABSTRACT

The chiral analysis of enantiomers is important because bioactivity can depend strongly on stereochemistry as ligand-protein binding motifs are typically chiral. Ion mobility mass spectrometry-based methods are emerging for the rapid and sensitive chiral analysis of molecules. However, such methods are typically limited by the use of metal-bound trimers, which can be challenging to form owing to ion suppression and the need for extensive pre-screening experiments to identify suitable metal ions. Moreover, the chiral separation of very small molecules, such as cysteine and proline, using ion mobility has remained challenging. Here, using electrospray ionisation high-resolution differential ion mobility mass spectrometry (ESI-DMS-MS), we demonstrate that the enantiomers of benchmark amino acids as small as proline can be rapidly distinguished without the use of metal ions for the first time. ESI-DMS-MS of proton-bound diastereomeric dimer complexes, containing enantiomers of amino acids and a 'chiral selector' (*N-tert*-butoxycarbonyl-*O*-benzyl-L-serine; BBS) corresponding to $[L/D\text{-X(BBS)}+H]^+$ (X = cysteine and proline) resulted in the separation of *L* and *D*-enantiomers. By use of DMS-MS and standard solutions of chiral mixtures, these data indicate that the enantiomeric excess of proline can be accurately quantified by differential ion mobility mass spectrometry. Overall, these results provide further evidence that DMS-MS can be used for the rapid and accurate 'metal-ion free' chiral analysis of many other biologically important molecules.

1. Introduction

The biological activity of enantiomers can differ significantly because ligand-protein binding motifs are typically chiral [1–3]. Thus, enantiomeric analysis of small molecules is invaluable in the quality control of chemical catalysis and pharmacological applications [4]. In the development of analytical methods for chiral analysis, amino acids are typically used as benchmark molecules to optimise and compare the performance of different approaches [5]. For enantioselective separations, Pirkle's rule stipulates that an asymmetric chiral environment is required to promote diastereomeric interactions between an enantiomer and a chiral

* Corresponding author.
E-mail address: w.donald@unsw.edu.au (W.A. Donald).

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environment [6]. For chiral analysis, mass spectrometry (MS) is particularly promising owing to its ability to rapidly detect analytes at trace levels directly from chemical mixtures with high sensitivity [1,2,7]. A relatively well-established MS method for chiral discrimination is to use collision-induced dissociation (CID) of diastereomeric, non-covalently bound complex ions that contain usually two 'chiral selector' molecules and one 'analyte enantiomer'. Given that such diastereomeric complex ions can have binding energies that depend on the chirality of the analyte, the relative fragment ion abundances can be used for chiral discrimination [8]. However, for proton-bound dimers of amino acids, the binding energies of all diastereomeric complex ions reported to date do not depend sufficiently on the chirality of the analyte for enantiomeric recognition by CID-MS [9,10].

Ion mobility mass spectrometry (IM-MS) can be used for the rapid and direct chiral analysis of molecules in diastereomeric complex ions that usually contain one metal ion, two chiral selector molecules and one chiral analyte molecule. In IM, ions are separated based on their difference in ion mobility through a carrier gas, under a constant low electric field prior to detection by MS [8,11]. Ion mobility depends on a number of factors, including the mass, charge, and collision cross-section (Ω) of the ion; the temperature, pressure, and identity of the carrier gas; and the electric field strength [1,2,12]. For resolving diastereomeric complex ions, the collision cross-sections must depend significantly on the analyte enantiomer. However, smaller chiral molecules cannot typically be enantiomerically resolved because the diastereomeric interactions of smaller molecules with a chiral selector impact the Ω of diastereomeric cluster ions to a lesser extent than larger analyte molecules of a similar type (*e.g.* amino acids) [13]. For example, to obtain selectivity in chiral analysis using IM-MS, it is important that diastereomeric complexes with exceedingly minor differences in collision cross-sections can be resolved [2].

Chiral analysis of small molecules has been performed previously by travelling wave ion mobility spectrometry (TWIMS) and cylindrical-based high-field asymmetric waveform ion mobility spectrometry (FAIMS) [5,14]. By forming metal-bound *trimer* complex ions, a molecule as small as threonine could be resolved by TWIMS [5], and proline using FAIMS [14], thus making proline the smallest molecule that has been enantiomerically resolved using any ion mobility-based method to date. In these experiments, many different trimer complex ions containing appropriate metal ions and chiral selector molecules must be systematically screened to identify appropriate combinations that can enable successful chiral resolution. Although these approaches are powerful for chiral separations, smaller amino acids cannot be readily resolved. For example, the use of TWIMS-MS did not result in the resolution of cysteine enantiomers [5]. Moreover, the use of metal-bound trimers is limited by the need to: (i) optimise concentration ratios between the analyte and chiral selector; and (ii) identify the optimal metal ion that gives the highest degree of enantioselectivity. By use of higher resolving power instrumentation for chiral separations, it should be possible to directly separate proton-bound diastereomeric dimers for chiral analysis of small molecules to eliminate any limitations associated with the use of metals and trimer complexes.

Recently, high-definition differential ion mobility spectrometry (DMS), also known as planar FAIMS that is operated using a high concentration of low-molecular weight carrier gases (He or H_2), has been used to separate ions with a resolving power as high as ~500, which is amongst the highest reported for any ion mobility-based method [15]. In DMS, ions travelling between two electrode plates are separated based on the difference in ion mobility under a high compared to a low electric field (E) by application of a high-field asymmetric alternating current waveform and a direct-current compensation voltage (CV) between the two electrodes [15,16]. Ions of a specific CV value, corresponding to a 'compensation electric field' (E_C), can be selectively transmitted through the device and into a mass spectrometer for detection [14,16,17]. By measuring the abundance of ions as a function of E_C, a DMS-MS spectrum can be obtained. DMS-MS has the advantage that the mobility of ions under the influence of a high electric field depend nonlinearly on the electric field strength, which results in ion separation being more orthogonal to ion mobility methods that operate using lower electric fields [18]. Moreover, ion separation processes occur in low milliseconds which is many orders of magnitude faster than traditional chromatography-based chiral separations. Although chromatographic methods for chiral separation are well established and enable preparative separations, methods based on ion mobility and mass spectrometry can be advantageous for analytical separations [3].

Recently, we demonstrated that high-definition DMS-MS can be used to resolve the proton-bound diastereomeric dimers of relatively large amino acids (*i.e.* tryptophan and phenylalanine) [19] that are: (i) 40–80% larger than proline in terms of mass; (ii) 60–90% larger than cysteine in terms of the number of atoms; and (iii) 13–21% larger than proline in terms of collision cross-section for the protonated amino acids (N_2; 200 V/cm) [20]. Here, this approach can be used for the rapid chiral recognition of enantiomers of amino acids as small as proline without using metal ions. The performance of this approach was benchmarked to that of the more well-established collision-induced dissociation MS method using proton and metal-bound dimers.

2. Experimental

2.1. Materials

L-Cysteine, D-cysteine, L-proline, and D-proline, and *N-tert*-butoxycarbonyl-*O*-benzyl-L-serine (BBS) were purchased from Sigma (St Louis, MO, USA) and used without further purification. Lithium chloride, sodium chloride, magnesium nitrate, and copper (II) sulphate were purchased from Ajax Finechem (Taren Point, NSW, Australia); potassium carbonate was purchased from Chem Supply (Gillman, SA, Australia); rubidium chloride was purchased from Sigma (St Louis, MO, USA); calcium chloride was purchased from VWR Chemicals (Leuven, Belgium); chromium (III) bromide hexahydrate, and lanthanum (III) chloride hydrate were purchased from Strem Chemicals (Newburyport, MA, USA). Methanol was obtained from Scharlau (Sentmenat, Spain). Deionized water (18 MΩ) was obtained using a water purification system (MilliQ, Merck, Darmstadt, Germany).

2.2. Diastereomeric complexes

Electrospray ionisation (ESI) solutions containing equimolar concentrations of the amino acid (200 μM) and BBS (200 μM) were prepared in 99:1 methanol:acetic acid for MS experiments. Racemic solutions were prepared using stock solutions of *L*- and *D*-enantiomers (200 μM each), and BBS (400 μM) in 99:1 methanol:-acetic acid. To obtain a calibration curve, concentrations of *L*- and *D*-enantiomers were adjusted such that the total concentration of *L*- and *D*-enantiomers was 400 μM. For metal-bound diastereomeric complexes, stock solutions of the metal salts were prepared in 18 MΩ water. To form metal-bound dimers for CID experiments, solutions containing the amino acid, BBS, and the metal salt were optimised in solutions of 99:1 methanol:acetic acid to maximise dimer ion signal (Table S1, Supplementary Information).

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