Published in final edited form as:

Chem Commun (Camb). 2018 August 21; 54(68): 9398–9401. doi:10.1039/c8cc03790f.

Chiral Separation of Diastereomers of the Cyclic Nonapeptides Vasopressin and Desmopressin by Uniform Field Ion Mobility Mass Spectrometry

Shawn T. Phillips^a, James N. Dodds^a, Berkley M. Ellis^a, Jody C. May^a, and John A. McLean^a

^aDepartment of Chemistry, Center for Innovative Technology, Vanderbilt Institute of Chemical Biology, and Vanderbilt Institute for Integrative Biosystems Research and Education, Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN, 3726, United States

Abstract

In this study ion mobility-mass spectrometry (IM-MS) is used to distinguish chiral diastereomers of the nonapeptides desmopressin and vasopressin. The differences in gas phase cross sectional area (*ca.* 2%) were sufficient to directly resolve the enantiomers present in a binary mixture. Results from computational modeling indicate that chiral recognition by IM-MS for nonapeptides is possible due to their enantiomer-specific conformations adopted in the gas-phase, namely a compact ring-tail conformer specific to the L-enantiomer forms.

Chiral recognition plays a significant role in determining the biochemical activity of pharmaceuticals and their protein targets.¹ For example, (*R*)-thalidomide was widely prescribed due to its therapeutic properties for alleviating the symptoms of morning sickness, while (*S*)-thalidomide was found to produce birth defects in newborns.² While most chiral molecules are separated and characterized by chromatographic methods, these assays can require substantial analysis time and sample derivatization.³ Recognizing the significant impact of chirality on biological activity, discovering rapid and selective methods to separate and evaluate chiral compounds is imperative in quality control efforts related to pharmaceutical production.⁴

Ion mobility-mass spectrometry (IM-MS) is an integrated analytical technique that separates gas phase ions based on their size (collision cross section, CCS) and weight (mass-to-charge ratio, *m/z*).⁵ These separations occur in the millisecond (IM) and microsecond (MS) time scales and hence provide a unique and rapid 2-dimensional analytical separation. IM-MS has previously been shown to effectively separate and characterize isomers spanning many biological classes including carbohydrates, lipids, and fatty acids.^{6–8} Here, we utilize IM-MS to evaluate the stereochemistry of macromolecular diastereomers and explore the conformations of nonapeptides.

Correspondence to: John A. McLean.

Electronic Supplementary Information (ESI) available: The Supplemental Information includes a table of CCS values in nitrogen, helium drift spectra, fragmentation data, and hierarchical clustering.

Conflicts of interest

There are no conflicts to declare.

Nonapeptide hormones are a family of intrinsically disordered cyclic peptides whose structure consist of a nine amino acid chain comprised of a six-membered amino acid ring bridged by a cysteine-cysteine disulfide bond and a three amino acid side-chain, referred to as the tail (Fig. 1). The expression of nonapeptide hormones is conserved throughout both vertebrates and invertebrates where they function in a variety of biochemical roles including vasoconstriction (vasopressin) and reproduction (oxytocin) among others.⁹ Two of these nonapeptides are studied here and described in Table 1. Vasopressin (8L-arginine-vasopressin, 8L-VP) is a naturally-occurring hormone responsible for vasoconstriction and blood pressure control,¹⁰ and desmopressin (1-desamino-8D-arginine-vasopressin, 8D-DP), a synthetic derivative of vasopressin, has been used predominantly in the treatment of nocturnal enuresis.¹¹

Desmopressin varies structurally from vasopressin in two locations in the peptide sequence: (1) the absence of an amine group at the 1-Cys residue of the amino acid ring, and (2) D-stereochemistry at the 8-Arg residue. Enantiomeric forms of these nonapeptides can also exist depending on whether the 8-Arg residue is expressed with D- or L- stereochemistry, resulting in four distinct nonapeptides: 8L-arginine-vasopressin (8L-VP), 8D-arginine-vasopressin (8D-VP), 1-desamino-8D-arginine-vasopressin (8D-DP), and 1-desamino-8L-arginine-vasopressin (8L-DP).

In this study, ion mobility separations for the diastereomers of vasopressin and desmopressin were facilitated using a commercial drift tube IM-MS (Agilent 6560) equipped with an electrospray ionization source operated in positive ion mode.¹² Samples of each diastereomer and 1:1 mixture of the diastereomeric pairs of vasopressin and desmopressin were prepared at a concentration of 10 $\mu\text{g/mL}$ in ultra-pure water with 10 mM ammonium acetate added to provide a final solution with pH of *ca.* 6.5.[‡]

Arrival time distributions were converted to CCS space (See Supplemental Table S1), and the resulting CCS distributions are contained in Figure 2. Figure 2A depicts the protonated $[M+H]^+$ adducts for the diastereomers of desmopressin [(L)-green trace and (D)-purple trace] along with the corresponding 1:1 mixture (grey dotted line). The IM spectra were obtained at an optimal separation drift field of 17 V/cm.¹³ Protonation is expected to occur at the 8-arginine position as this amino acid has the highest pKa of all residues in each molecule. IM distributions for 8L-DP and 8D-DP produce a unique single CCS distribution for each diastereomer (CCS of 308 $\text{\AA}^2$ and 314 $\text{\AA}^2$, respectively). This difference in CCS (*ca.* 2%) is substantially larger than other studies of diastereomers observed in our laboratory (*ca.* 0.4% difference in CCS), which is enough to allow for separation of both molecules in a two component mixture (grey dotted trace).¹⁴ The significant difference in CCS for the diastereomers of desmopressin in comparison to our previous small molecule studies is most likely attributed to significant differences in the gas-phase structures of these two isomers. Specifically, we hypothesize that 8L-DP can adopt a structure with a smaller cross section due to intermolecular folding of the tail with the ring system, whereas the 8D-DP

[‡]To check the possible effects of pH on the structures and their corresponding spectra, samples were prepared and analysed at pH 6.5, 5.8, and 5.5 using ammonium acetate buffer and formic acid. There was no change in the observed spectra for any of the diastereomers over this pH range.

diastereomer predominantly exists as an extended conformer with a larger (*ca.* 2%) CCS distribution. Previous IM studies have indicated that lasso peptides can exhibit multiple and complex conformations in the gas-phase.¹⁵ Figure 2B contains the CCS distributions for the protonated (M+H)⁺ adducts for the diastereomers of vasopressin [(L)-green trace and (D)-purple trace] along with the corresponding 1:1 mixture (grey). While the diastereomers of desmopressin each consist of single Gaussian arrival time distributions as noted in Figure 2A, the spectra for the diastereomers of vasopressin (Figure 2B) exhibit a single unique distribution for this 8D-VP, whereas two peaks with corresponding CCS of 303 Å² and 310 Å² (*ca.* 2% difference) are noted for 8L-VP. Interestingly, the larger conformer of 8L-vasopressin (CCS 310 Å²) aligns with the cross section for the 8D-VP, suggesting a structural relationship between these two molecules. These conformational observations were also noted in helium drift gas, and hence are not buffer gas specific (see Supplemental Figure S1).

To ensure the two distinct distributions of 8L-VP were not a result of impurities in the sample, mobility-resolved fragmentation experiments of the two peaks were conducted (see Supplemental Figure S2). Mobility-aligned fragmentation spectra obtained at 60 V (laboratory frame) illustrate that both conformations contained identical fragmentation profiles and fragment relative abundances suggesting they are the same molecule, and not a contaminant. While the differences in stereochemistry for 8L-VP and 8D-VP do not produce different fragmentation spectra (see Supplemental Figures S3 and S4), this information provides additional confidence that the two ion mobility distributions observed for 8L-VP originate from a molecule with the same chemical formula and not a degenerate artifact of the analyte, such as a multimeric species. To further demonstrate the purity of the 8L-VP conformers, an additional sample of 8L-VP from a separate vendor was analysed and provided identical CCS distributions as those noted in Figure 2B (Supplemental Figure S5). Furthermore, rotating-frame nuclear Overhauser effect correlation spectroscopy (ROESY) ¹H NMR spectra were also acquired and demonstrate that both samples from the manufacturer have not been cross-contaminated (Supplemental Figures S6 and S7).

The sole structural distinction between the diastereomers of desmopressin and vasopressin are the changes in chirality at 8-Arg (colored triangles in Fig. 2A/B) and the corresponding deamination. This conversion in stereochemistry for the diastereomers of desmopressin under these conditions results in each molecule possessing a different experimentally observed cross sectional area which suggests a distinct three-dimensional gas phase structure is adopted for each. While similar behavior is observed for the diastereomers of vasopressin (*i.e.* a different cross section for each diastereomer), there is also the appearance of two peaks for 8L-VP suggesting that there are two unique conformations for this diastereomer.

A significant number of publications in the field of NMR and molecular modelling have been carried out on vasopressin and desmopressin and provide insight into the structural characteristics of these compounds. Of note, the tail-ring interactions (cartoon depicted in Figs. 2,3) and different ring folding conformations have been proposed to account for several different possible conformations for both desmopressin and vasopressin.¹⁶ We hypothesized that these reported interactions were contributing to the two distinct conformations of 8L-VP observed in the ion mobility spectra and we used computational models to comprehensively

investigate our experimental measurements. Each vasopressin and desmopressin diastereomer was subjected to simulated annealing calculations in Molecular Operating Environment (MOE) using the MM94ffx force field in order to generate 3,000 low energy conformations. These conformations were then used to calculate CCS values in nitrogen drift gas using the Projected Superposition Algorithm (PSA).¹⁷ Although experimental CCS measurements obtained were slightly smaller (*ca.* 1.7% different) than the calculated CCS values, the conformational separations were conserved using this approach. The theoretical CCS density for vasopressin represented in Figure 3C articulates the respective separations in that two conformational distributions are observed for 8L-VP and one primary distribution for 8D-VP. 8L-VP exhibits an increased number of conformations at lower CCS values, which is indicative of the propensity 8L-VP to stabilize the basic arginine residue and tail moiety within the ring (Figure 3A). This folded conformation appears to solely be present in the 8L-VP generated conformations, in addition to the more extended conformer wherein the tail system is interacting with glutamine on the outside of the ring (Figure 3B) which is predominantly observed in 8D-VP. Furthermore, heavy atom root-mean-square-deviation (RMSD) hierarchical clustering reflected these analyses, wherein calculations for 8L-VP returned two distinct families of populations representing the folded and extended conformations which comprise 30% and 27% of the total generated conformations (N=3,000), respectively (Supplemental Figure S8). RMSD calculations for 8D-VP and desmopressin diastereomers reflected one prominent family of the extended conformation representing ~40% of the structures with the remaining families representing sparse structural populations. The described computational models infer the chirality associated with the arginine residue gives rise to different low energy gas phase populations within the vasopressin and desmopressin systems (Figure 3C and Supplemental Figure S9). Specifically, a tail-ring conformer at lower CCS and an extended-tail conformer at higher CCS.

Because of the potential effect of chirality in biological systems as well as in the field of pharmaceuticals, there is a increasing need to discover new methods to rapidly evaluate chiral systems. In this study we have illustrated the use of IM-MS to recognize macromolecular structural differences between the diastereomers of desmopressin and vasopressin. Indeed, recent reports have demonstrated that chiral selectivity can be achieved in ion mobility for multimeric complexes such as analyte dimers, trimers, and tetramers.^{18–20} The ability of IM-MS to recognize the presence of a mixture of diastereomers as well as conformations in these molecules suggests its application to more extensive areas, including the characterization of the chiral distribution of pharmaceuticals following synthesis and purification.^{21,22}

We gratefully acknowledge Prof. Prasad Polavarapu and Dr. Donald Stec for their expertise and contributions towards chiral recognition and structural determination. This work was supported in part using the resources of the Center for Innovative Technology (CIT) at Vanderbilt University. Financial support for this work was provided by the National Institutes of Health (NIH NIGMS R01GM092218, NIH NCI 1R03CA222452–01, and S10 RR019022) and the U.S. Environmental Protection Agency (EPA) under Assistance Agreement No. 83573601. This work has not been formally reviewed by the EPA and EPA does not endorse any products or commercial services mentioned in this document.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig.1. Chemical scaffold of nonapeptide hormones desmopressin and vasopressin. The ring system is indicated in yellow, and the tail region is highlighted in blue.

Fig.2. Ion mobility spectra of the protonated nonapeptides (desmopressin and vasopressin, **A** and **B**, respectively) with representative depictions of intramolecular folding (at right in each panel).

Fig.3.

Structural representations of theoretical conformations contributing to the observed CCS profiles of 8L-vasopressin. **(A)** A compact CCS conformation observed as the tail arginine residue (blue) is stabilized within the ring (yellow). **(B)** An expanded conformer is observed wherein the tail and arginine residue interact on the periphery of the ring with glutamine. **(C)** Distribution of the number of theoretical conformations across calculated CCS values. The modelling results exhibit a broader ensemble of structures for 8L-VP extending to a lower

CCS than is observed for 8D-VP. Both experimental and theoretical values are reported for nitrogen buffer gas.

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Table 1

Abbreviations and designations of chirality for diastereomers in this work.

Nonapeptide	Abbreviation	Cysteine 1	Arginine 8
Vasopressin	8L-VP	–H	L
	8D-VP	–H	D
Desmopressin	8L-DP	–NH ₂	L
	8D-DP	–NH ₂	D