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Short communication

Determination of related substances in disulfide-rich peptide ziconotide by establishing tandem mass spectrometry methods

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Currently, several disulfide-rich peptides have been officially approved as therapeutic medicines, such as ω-conotoxin ziconotide [1], guanosine analogue linaclotide, and plecanatide. Peptides are predisposed to generate related substances (also termed as structurally related impurities) including amino acid deletion/insertion, diastereoisomerization, deamination/amination, oxidation, and succinimidation etc. during synthesis, storage, and transportation processes [2]. In addition, peptide degradation may happen during many more aspects/processes (e.g., reconstitution, transfer processes in instruments, and work-up procedures), which increases the difficulties for data interpretation. For disulfide-rich peptides, related substances associated with disulfide bond modifications (e.g., disulfide bond deletion or exchange) are of particular concern as they may significantly affect drug stability and positive results in preclinical research [3]. Recently, liquid chromatography-high resolution mass spectrometry (LC-hrMS) and ion mobility-MS (IM-MS) methods were developed for related substances determination and conformational purity assessment of hepcidin containing four disulfide bonds [4]. However, the impurities associated with disulfide bond deletion or isomerization were not reported, nor was the corresponding characterization strategy. Peptides containing two or three disulfide bonds have advantages of superior metabolic stability and biological activity, and thus are the primary objects in the pharmaceutical market and research and development stage currently [5].

Ziconotide is the first member receiving approval for intrathecal therapy of severe chronic pain in the disulfide-rich neuroactive conopeptide family. Its amino acid sequence is CKGKGAKCSRL-MYDCCTGSCRSGKC-NH_2, in which three disulfide bonds are formed with connectivity of C1−C16, C8−C20, and C15−C25.

For characterizing related substance, the conventional method by fitting the LC retention time of the synthetic standard is no longer applicable because of the time-consuming and resource-intensive nature of synthesizing disulfide-rich peptide standards. In this work, related substances in a ziconotide study material were firstly separated by an optimized LC-MS method (Fig. S1) and the detailed information (e.g. retention times, charge states, and *m/z* values) are listed in Table S1. The details of materials and methods used in this study are shown in the Supplementary data. To avoid wrong interpretation of the type and amount of degradation impurities, ziconotide solution was freshly prepared prior to analysis. Related substances determination for ziconotide poses more challenges because the constraint of disulfide bonds makes it hard to obtain sufficient fragment information for *de novo* sequencing peptide impurities directly by tandem MS. Therefore, 1.5 h of reduction reaction was performed, breaking disulfide bonds after carefully optimization (Fig. S2).

Two related substances with mass shift of +16 Da were observed at retention times of 12.6 and 13.1 min (Fig. S1). Through MS^n (Fig. S3), both of them were ascertained to be oxidation form at the 12th position (e.g., methionine residue, M), thus termed as [Mox^{12}]ziconotide and [Mox^{12}]ziconotide isomer. The oxidation of methionine residue generates methionine sulfoxide, in which a stereogenic sulfur center is formed. These two diastereomers were clearly separated though they have similar partition coefficient in LC. A related substance with a mass shift of −32 Da was detected at retention time of 38.3 min (Fig. S1). Only 4 Da, instead of 6 Da, mass increase was observed after complete disulfide bonds reduction, indicating a disulfide bond deletion impurity. Upon collision-induced dissociation (CID) (Fig. S4A), this substance was identified as [dehydro-A^{16}]ziconotide, in which a cysteine (Cys) moiety at 16th position is replaced by a dehydroalanine moiety. [dehydro-A^{16}]ziconotide is produced by the β-elimination of Cys residue at the 16th position after cleavage of disulfide bond C1−C16. A related substance with a mass shift of −87 Da at retention time of 48.3 min (Fig. S1) was identified as ziconotide-9S, in which a serine moiety is deleted, based on MS^2 spectrum (Fig. S4B). A related substance with a mass shift of +71 Da at retention time of 31.2 min (Fig. S1) was

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identified to be alanine (Ala) or β-Ala residue insertion at the 10th position by MS^2 and MS^3 (Fig. S5). Given this position is not adjacent to an Ala residue, and β-Ala is commonly found existing as an impurity in the material of fluorenylmethyloxycarbonyl (Fmoc) protected amino acids, this substance was further judged to be ziconotide + βA10. Two ziconotide isomers (termed as isomer 1 and isomer 2 according to the order of elution time) were detected based on accurate mass and MS^n. For quantitative determination, a calibration curve was established (Fig. S6). Mass fractions and corresponding expanded uncertainties of related substances in the ziconotide study material were quantitatively determined (Table S2).

For a disulfide-rich peptide, disulfide bond exchange leads to conformation variation, which can be determined by IMS [4]. In this study, ziconotide isomer shows same ion mobility behavior as ziconotide in a mobilogram (Fig. S7), indicating the absence of disulfide exchange impurity. Complete reduced product of disulfide bond exchange isomer should have same retention time as that of

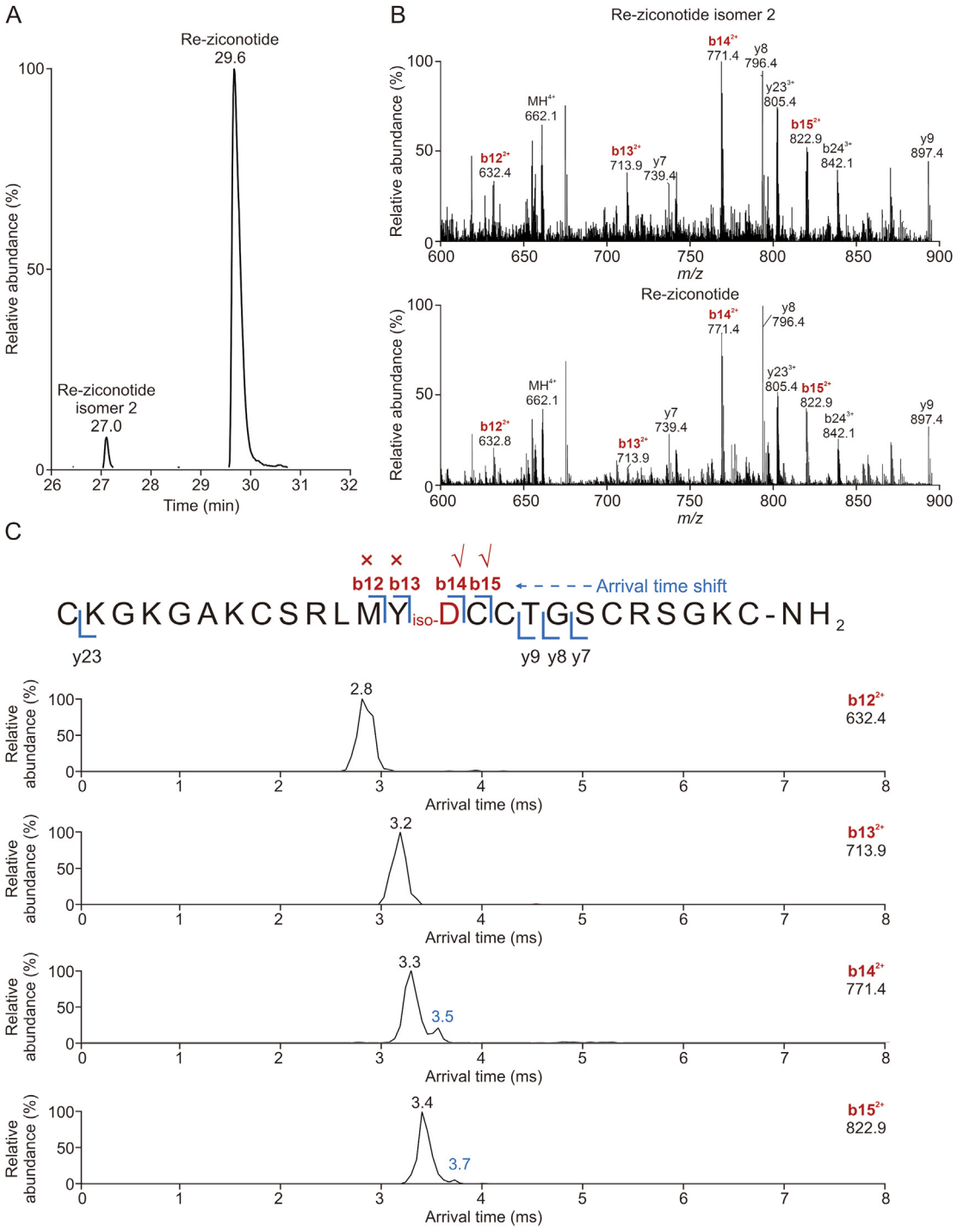

Fig. 1. Localization of isomerization site of [iso-Asp[14]]ziconotide. (A) Extracted ion chromatogram. (B) Tandem mass spectra of re-ziconotide isomer 2 (up) and re-ziconotide (down). (C) Isomerization site located by arrival time shift (√: shift; ×: without shift). Asp: aspartic acid.

ziconotide, because their structures are exactly same. The possibility of disulfide bond exchange impurity was further excluded because different retention times of complete reduction products of ziconotide and its isomer were observed (Fig. 1A). In this study, an MS^2-IMS method was established for locating the isomerization site of ziconotide isomer 2, the impurity with highest level. First, reduced ziconotide isomer 2 and reduced ziconotide were separated by LC. Then, a series of their fragments, which are extended from N-terminus and C-terminus (termed as b and y ions), (Fig. 1B) generated upon CID were analyzed by IMS. The b/y fragments containing the isomerization site, leading to conformational differences, could result in mobility difference during IMS analysis. The b12 and b13 ions from ziconotide isomer 2 and ziconotide have same arrival time distributions. In contrast, b14 and b15 show arrival time changes (Fig. 1C). The arrival time shift of fragmentation ions begins from b14, indicating the isomerization site is located at the 14th position. This substance could possibly be derived from the substitution of aspartic acid (Asp) residue by D-Asp or iso-Asp residue. Considering iso-Asp version of ziconotide is one of the thermal degradation products generated by hydrolysis of succinimide, this substance was finally judged to be [iso-Asp^{14}] ziconotide.

In summary, novel methods based on tandem MS have been established, characterizing related substances in a complex disulfide-rich peptide. This is also the first study to comprehensively determine related substances in a ziconotide material. By the LC-$hrMS^2$ method developed, seven ziconotide-related impurities were separated and characterized. A total mass fraction of related substances in the ziconotide study material was determined to be 206 mg/g ± 28 mg/g ($k = 2$). Among these impurities, ziconotide isomer 2 stands out as a critical component (>100 mg/g), which significantly impacts the peptide purity and quality of the ziconotide. An MS^2-IMS method was developed to locate the isomerization site for a disulfide-rich peptide isomer without requiring synthetic standards. Finally, ziconotide isomer 2 was identified as [iso-Asp^{14}]ziconotide. Despite [iso-Asp^{14}]ziconotide has not been reported possessing toxicity or activity, the presence of such high level impurity still leads to concerns about the efficacy of ziconotide due to low purity. Because the level of ziconotide isomer 1 is lower than the reporting threshold (1 mg/g) guided by pharmacopeias, locating its isomerization site was not attempted. Although related substances in the ziconotide study material were characterized based on MS^2 methods in this study, "separation-preparation-identification" strategy is still recommended for new drug application. The results in this work could complement the impurity profiles of ziconotide to ensure pharmaceutical product quality. The methods established here could also benefit for in-depth research on related substances for other disulfide-rich peptides and peptide purity assignment using mass balance approach (e.g., summation of impurities).

CRediT author statement

Yuya Cheng: Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Visualization, Writing - Original draft preparation; **Peize Wu:** Methodology, Formal analysis, Data curation, Visualization, Investigation; **Peng Xiao:** Methodology, Formal analysis, Data curation, Investigation, Resources; **Ming Li:** Conceptualization, Methodology, Validation, Supervision, Project administration, Funding acquisition, Writing - Reviewing and Editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2024.101037.

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