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Fragmentation of peptides with intra-chain disulfide bonds in triple quadrupole mass spectrometry and its quantitative application to biological samples

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ABSTRACT

A growing number of peptides are being used today in bioanalytical laboratories. Because of this, there is an increasing interest in the development of highly sensitive, specific and robust liquid chromatography/tandem mass spectrometry (LC/MS/MS) assays for the quantitative analysis of peptides in biological samples. Among the mass spectrometers previously used for peptide quantification, triple quadrupole mass spectrometers are generally not considered the instrument of choice. With this instrumentation, collision cascades or multiple fragmentations tend to generate multiple peaks that have weak intensities. This leads to a loss in detection sensitivity. However, in cases where immonium product ions were formed in abundance, it was found that peptide quantification succeeded. A common feature of these peptides is their intra-loop structure. To elucidate the usefulness of this feature in fragmentation, several peptide analytes with intra-chain disulfide bonds were investigated in this study, including a newly synthesized analog having a single amino acid substitution. The results presented here indicate that abrupt bond cleavage from the intra-loop structure of peptides could be one of the premises for intense immonium ion generation. In contrast, any preferential cleavage of peptide bonds (e.g., proline effect) that gives rise to a linearized sequence would break the intactness of the loop and prevent it from completely dissociating. In addition, the utilization of immonium product ions in LC/MS/MS was demonstrated for the determination of peptides with intra-chain disulfide bonds in biological fluids.
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1. Introduction

Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS), in providing a highly sensitive, specific and robust technique, has had a great impact on the quantitative analysis of a wide range of compounds. However, it is generally believed that the use of LC/MS/MS to detect and quantify peptides and proteins, which comprise important classes of drugs and biomarkers, is difficult [1]. Thus, bioassays, such as enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays (RIAs), have been traditionally employed. In recent years, interest in the application of LC/MS/MS toward peptide quantification has been growing, due to the increasing number of newly discovered peptides and proteins [2–4].

Among the variety of mass spectrometers used in the past for peptide quantification [5–8], triple quadrupoles are not as frequently recommended [9], despite the fact that they are currently the most common mass analyzers and have made a great historical contribution to mass detection. The performance of a mass spectrometer depends significantly on its fragmentation process. In the multiple reaction or selected reaction monitoring (MRM or SRM) modes of triple quadrupoles, fragments are derived from a collision cascade or multiple-step fragmentation [9]. During these stages, protonated ions undergo collisions and can further fragment, which tends to generate a large number of product ions at relatively low abundances. These standard modes of backbone cleavage and the resulting series of ions (so-called sequence tags [10]) accompanying de novo tools have proven extremely effective in the mass spectrometric sequencing of peptides [11,12]. However, the absence of dominant product ions does not satisfy the requirements of peptide quantification. Sensitive peptide analysis has been reported, as in the demonstration of structurally distinctive immonium ions that were formed in abundance. This was achieved via the combination of a- and y-type cleavages during secondary fragmentation [13]. Murao et al. have developed a simple and sensitive method for the determination of hepcidin-25 using the immonium product ion of phenylalanine residue [1]. Recently, Bredehoft et al. have applied proline immonium ions to quantify the human insulin-like growth factor-1 [14]. Interestingly, these peptides have the same intra-loop feature that is linked by

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disulfide bonds. Peptide bonds within the intra-loop are thought to be resistant to fragmentation under low-energy collision conditions [15,16], whereas high collision energy can lead to the cleavage of the intra-loop into immonium ions [14]. Thus, the use of immonium ions might provide a potential pathway for the quantitative analysis of peptides with intra-chain disulfide bonds. However, not all peptides with intra-loops are eligible for triple quadrupole mass detection that employs immonium ions. For example, Liu et al. did not find any intense immonium ions during the quantification of eptifibatide, a cyclic heptapeptide [17]. Therefore, the gas-phase fragmentation process of intra-loop peptides in triple quadrupoles deserves further investigation.

In this report, several oligopeptides, including desmopressin and eptifibatide, were selected to investigate the fragmentation of peptides with intra-chain disulfide bonds in a triple quadrupole mass spectrometer. Stepwise increments in collision energy were applied to reveal the optimal conditions of intra-loop dissociation and immonium ion generation. To further elucidate the eligibility of triple quadrupoles for the sensitive detection and reliable quantification of peptides, we also introduced EP-V6, which is a peptide analog of eptifibatide that has a single amino acid substitution. Finally, an investigation into the suitability of using immonium product ions in LC/MS/MS was performed for the peptides in human plasma.

2. Experimental

2.1. Chemicals and reagents

Desmopressin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Eptifibatide was obtained from Shanghai GL Peptide Ltd. (Shanghai, China). EP-V6 (Mpr-Har-Gly-Asp-Trp-Val-Cys-NH_2 (Disulfide Bridge, Mpr1–Cys6), Mpr-β-mercaptopropionic acid [18], Har-homoarginine [18]) was originally designed by our lab and developed by Shanghai GL Peptide Ltd. (Shanghai, China; Fig. 1). EP-V6 is a synthetic analog of eptifibatide that has a valine substitution for proline at position 6. As shown in the figure, each peptide has an intra-chain disulfide bond.

Acetonitrile (ACN) and formic acid were analytical grade and were purchased from Sigma–Aldrich (Shanghai, China). Water was purified and deionized using a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Liquid chromatography and mass spectrometry

A Waters ACQUITY UPLC system and a Quattro triple quadrupole mass spectrometer (Milford, MA, USA) were used.

Liquid chromatography separations were performed on an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 mm × 50 mm; Waters, USA) at room temperature. The mobile phase consisted of solvent A (0.1% formic acid:water) and solvent B (0.1% formic acid:ACN). A linear gradient with a flow rate of 0.6 mL/min was applied in the following manner (duration in parentheses): B 5% (0 min) → 5% (0.2 min) → 95% (1.2 min) → 95% (1.4 min) → 5% (1.5 min) → 5% (1.6 min). The injection volume was 2 μL.

The mass spectrometer was operated with an electrospray interface set at unit resolution and in the positive multiple reaction monitoring (MRM) mode. The desolvation nitrogen flow was 800 L/h, and the cone nitrogen flow was 50 L/h. The source and desolvation gas temperatures were held at 100 and 350 °C, respectively. The electrospray capillary and cone voltages were optimized to 3.5 kV and 50 V, respectively. Data were collected and processed using MassLynx software from Micromass-Waters (Manchester, UK). The collision energy was monitored in steps of 5 eV from 0 to 70 eV, with all other settings held constant. The concepts of low- and high-energy were introduced to distinguish collision conditions that led to different fragmentation patterns [19]. In this article, they were defined as 0–30 eV and >30 eV, respectively. It is noteworthy that this subjective division was established on experimental results and employed for ease of data interpretation. This could be quite different from other divisions that have been previously suggested [20–23].

MRM transitions used for desmopressin and EP-V6 quantification were m/z 1070 → 120 (328) and m/z 834 → 159 (834), respectively. Among them, the transition from m/z 834 → 834, where the analyte was detected by means of a protonated molecule, is sometimes called a pseudo-MRM [24]. Experimental conditions were optimized with a 1000 ng/mL infusion solution using a 10 μL/min flow rate of solvents A:B (50:50), to give the maximum response of the most abundant product ion. The dwelling time was set at 200 ms.

2.3. Preparation of stock, infusion and working solutions

Individual peptides were accurately weighted. Stock solutions were prepared at 1 mg/mL by dissolving weighed peptides into the working solvent ACN:water (50:50, v:v) containing 0.1% formic acid. They were stored in brown glass tubes, which protected them from light, at −20 °C. The stock solution was diluted to 1000 and 10 ng/mL for the infusion and working solutions, respectively. These were stored at 4 °C.

2.4. Preparation and pretreatment of plasma samples

EP-V6 calibration standards were prepared by diluting the EP-V6 stock solution with human plasma on ice. Concentrations of the calibration standards were 2, 5, 10, 25, 50, 100 and 250 ng/mL (2.4–300 pmol/mL). Subsequently, 25 μL of each sample was transferred into a 2 mL 96-well plate, followed by addition of 100 μL of ACN. The plate was covered and vortexed for about 5 min. Afterward, the plate was centrifuged at approximately 1740 × g for 10 min at 5 °C. A volume of 120 μL of supernatant was transferred into a new plate. Subsequently, 50 μL of water was added to all of the wells.

3. Results and discussion

3.1. Desmopressin

In the full scan spectrum of desmopressin, the base peak at m/z 535 corresponds to its doubly charged ion, which was more abundant than its singly charged ion at m/z 1070 (data not shown). With the application of collision energy (CE) in stepwise increments, a series of product ion spectra generated from the doubly charged precursor ion was obtained. Among them, the spectra at CEs of 10, 15, 35 and 45 eV are selectively depicted in Fig. 2. As shown, the dominant ions at a CE of 15 eV were m/z 328 and m/z 742 (panel B). Their predominant appearance did not change within the CE range of 0–30 eV. However, this situation changed when the CE reached 35 eV (panel C). At this point, product ions in a lower mass range were formed (e.g., m/z 136, 120, 84 and 70). When CE rose further to 45 eV, these ions became significant, and the peaks at m/z 328 and 742 entirely disappeared (panel D).

Analysis of these results suggests that the ions at m/z 328 and 742 represent the linear part of desmopressin and the 20 member intra-disulfide loop, which is composed of six amino acids, respectively (Fig. S1). These were formed by cleavage of the X-Pro/Cys bond outside the loop. The propensity of the peptide bond to preferentially cleave adjacent to proline residues has been noted in a number of reports. This is commonly attributed to the relatively high proton affinity of proline (i.e., proline effect) [25–27]. In other

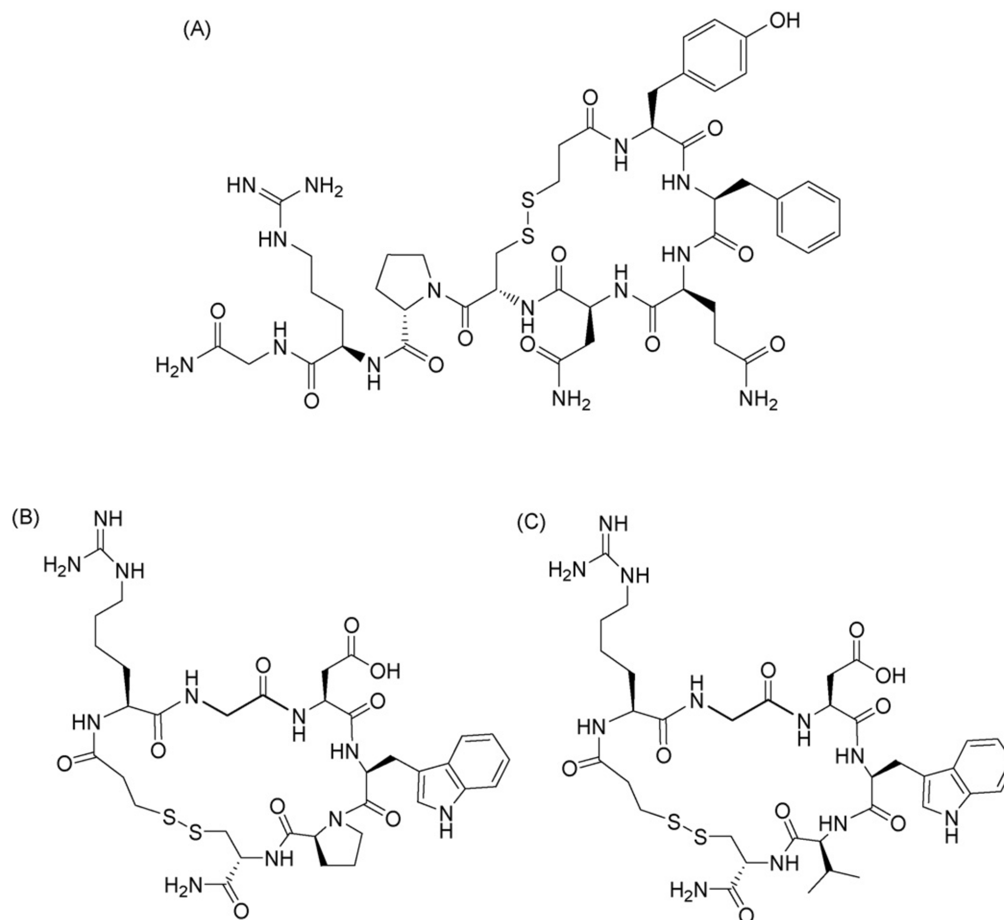

Fig. 1. Chemical structures of (A) desmopressin (MW = 1069), (B) eptifibatide (MW = 832) and (C) EP-V6 (MW = 834).

words, it is likely that there would not be any intense product ions preceding the dissociation of bonds within the loop if there was no proline in the linear part of the peptide. This is similar to cases shown for some linear peptides (see Section 3.4). The sustained presence of ions at m/z 328 and 742 over a wide range of CEs indicates the stability and intactness of intra-loop structures at low collision energies. Their resistance to fragmentation has been implied previously [14] and will thus not be explained here.

Further evaluation of newly generated product ions in the low-mass range, following high collision offset voltages, predominantly showed immonium ions. Among these ions, the most intense one was at m/z 120, which corresponds to the phenylalanine residue. Other immonium ions were derived from glutamine (m/z 84), asparagine (m/z 70) and tyrosine (m/z 136). The percentage of these four immonium ions compared to the total ion count was about 70% (Fig. 2, panel D). This implies their prevalence in the product ion spectrum of desmopressin under high-energy collisions. On the other hand, no prominent fragment ion series (e.g., b- and y-type ions), resulting from the conventional cleavage of peptide or disulfide bonds within the linearized sequence [28], were observed between intra-loop structure breakdown and immonium ion formation. This prevents the assumption of a linearization reaction in the fragmentation process of desmopressin. Taken together, these facts suggest that loop opening was probably achieved by an abrupt breakdown of precursor ions into low-mass fragments, reaching optimum CE rather than peptide linearization.

In the case of desmopressin, there was no evidence of the fragmentation pattern (e.g., multiple peaks with weak intensity) commonly observed for most peptides in triple quadrupoles [9,29]. On the contrary, immonium ions were obtained in abundance. To evaluate whether this type of fragmentation can be observed for other peptides with intra-chain disulfide bonds, eptifibatide and its analog EP-V6 were investigated.

3.2. Eptifibatide

Eptifibatide is a cyclic heptapeptide having one more intra-chain amino acid residue compared with desmopressin. Its lack of predominant product ions in triple quadrupole mass spectrometry has been previously reported [17]. Different CE levels (0–60 eV) were applied in the present work to reveal the conditions for its distinct fragmentation pattern. As shown in Fig. 3, panels A and B, the protonated eptifibatide molecule (m/z 832) was stable at CEs up to 40 eV, which is similar to the intra-loop part of desmopressin. Compared with desmopressin, the most striking difference was the formation of numerous fragments in the eptifibatide spectra as the CE was increased to 60 eV (panel C). After that, no significant changes in ion composition were observed in the spectra (data not shown). Product ions were also found in the lower mass immonium ion region (e.g., m/z 159, 84 and 171). However, none of these was strong enough for quantification, as earlier described. Interestingly, product ion series were identified for peaks spanning the middle mass range of the spectrum. For example, a complete set of b ions (i.e., m/z 242, 412, 469, 584 and 771), resulting from the amide bond cleavage at the C-terminal, is shown in Fig. 3, panel C.

Eptifibatide is similar to desmopressin in the composition of polar and charged amino acid residues. Differences in the fragmentation patterns of eptifibatide and desmopressin were probably caused by the position of proline residues within the peptides. As mentioned earlier, the amide bond adjacent to proline is a pre-

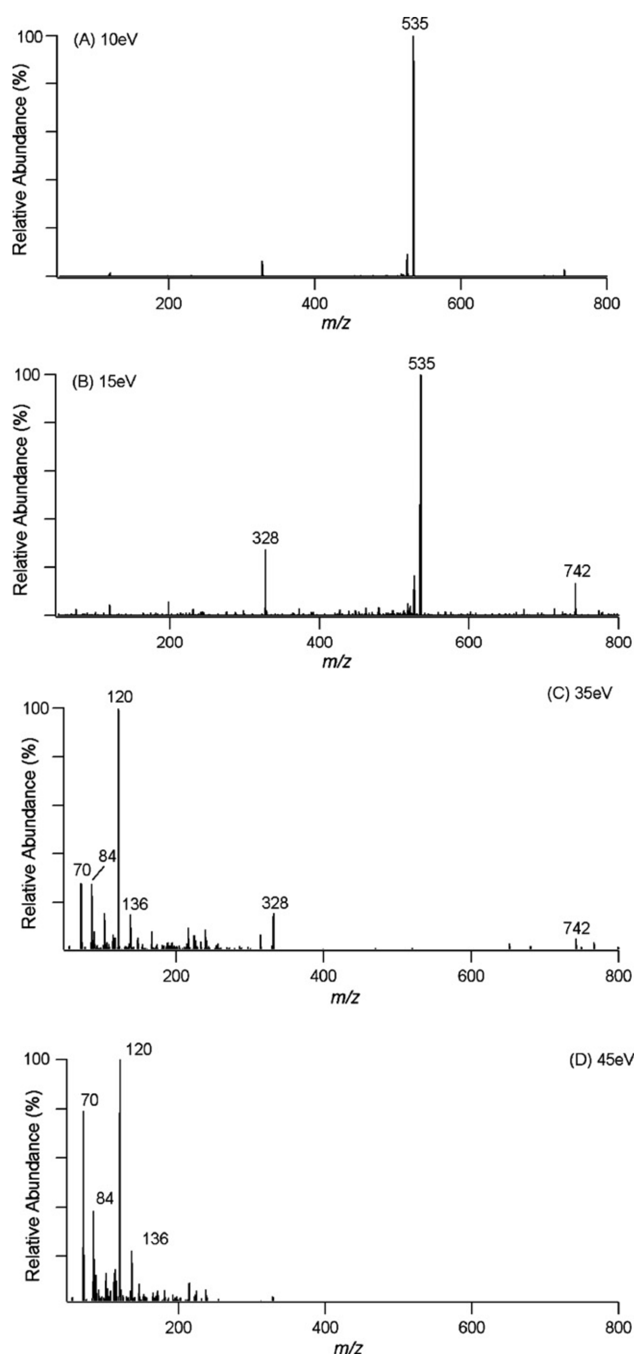

Fig. 2. Product ion spectra of desmopressin at collision energies of (A) 10 eV, (B) 20 eV, (C) 35 eV and (D) 45 eV.

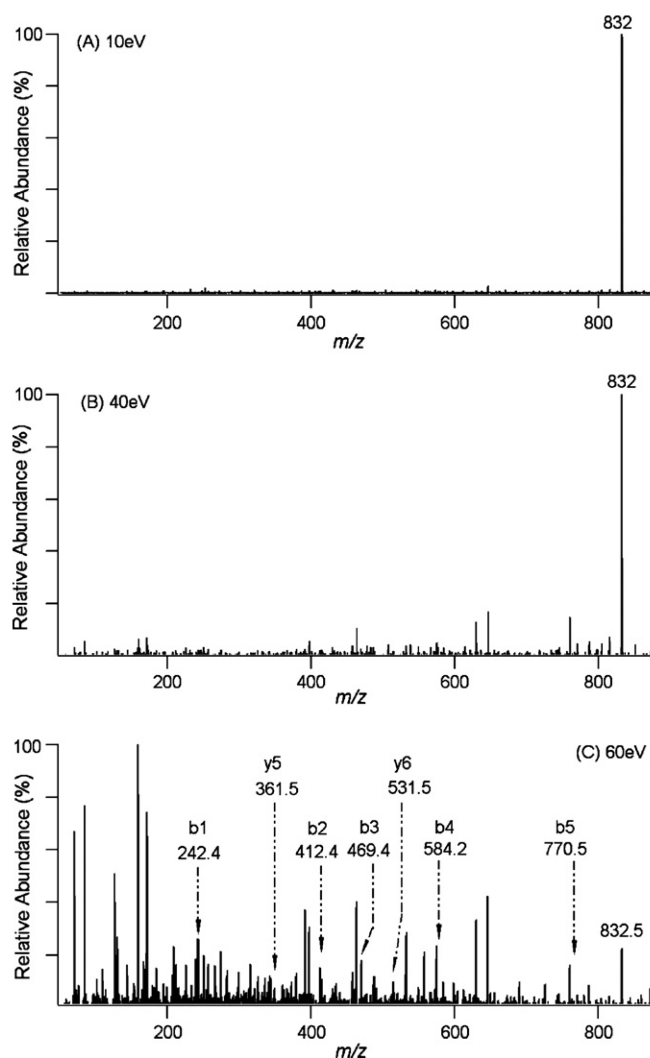

Fig. 3. Product ion spectra of eptifibatide at collision energies of (A) 10 eV, (B) 40 eV and (C) 60 eV with labeled b- and y-type ions.

determined cleavage point. Hence, cleavage of the amide bond, disruption of the stable ring structure and linearization of the peptide sequence could be promoted by the presence of a proline residue within the loop. Further fragmentation of the linearized peptide can occur, resulting in the formation of ions that correspond to conventional ion series derived from backbone cleavages. Therefore, the b- and y-type ions of eptifibatide imply the presence of a linearized peptide, followed by the cleavage of the intra-loop amide bond during the fragmentation process. Although this preferential cleavage adjacent to proline has facilitated the assignment of cyclic peptide sequences in other studies, due to the raised number of linear peptide fragments [28], a lack of strong product ions would virtually reduce the sensitivity of quantitative assays [30].

This finding clearly points out that the absence of loop breakdown prior to fragmentation can promote the quantitative analysis of peptides with intra-chain disulfide bonds. Thus, it was supposed that fragmentation following linearization would unlikely be observed, if no proline residues were present within the eptifibatide loop. Further experiments (e.g., no proline residue in the loop region) are required to provide more evidence either for or against this hypothesis.

3.3. EP-V6

To confirm the hypothesis that an intact intra-loop structure plays a vital role in the generation of immonium ions, eptifibatide was modified by replacing its proline residue with valine. Proline and valine have similar chemical compositions, and their difference in molecular weight is only 2 mass units. Thus, it can be assumed that the size and steric effects of this substitution are insignificant. The product ion spectra of EP-V6 are shown in Fig. 4. The peak intensity of the protonated molecule at m/z 834 was strong under low collision energies (panel A). Immonium ions (e.g., m/z 84, 127 and 159) appeared when the collision offset voltage reached 40 eV (panel B). Contrary to what was observed for eptifibatide, the intensity of these product ions continually increased with increasing CE (data not shown), achieving the largest and most dominant pres-

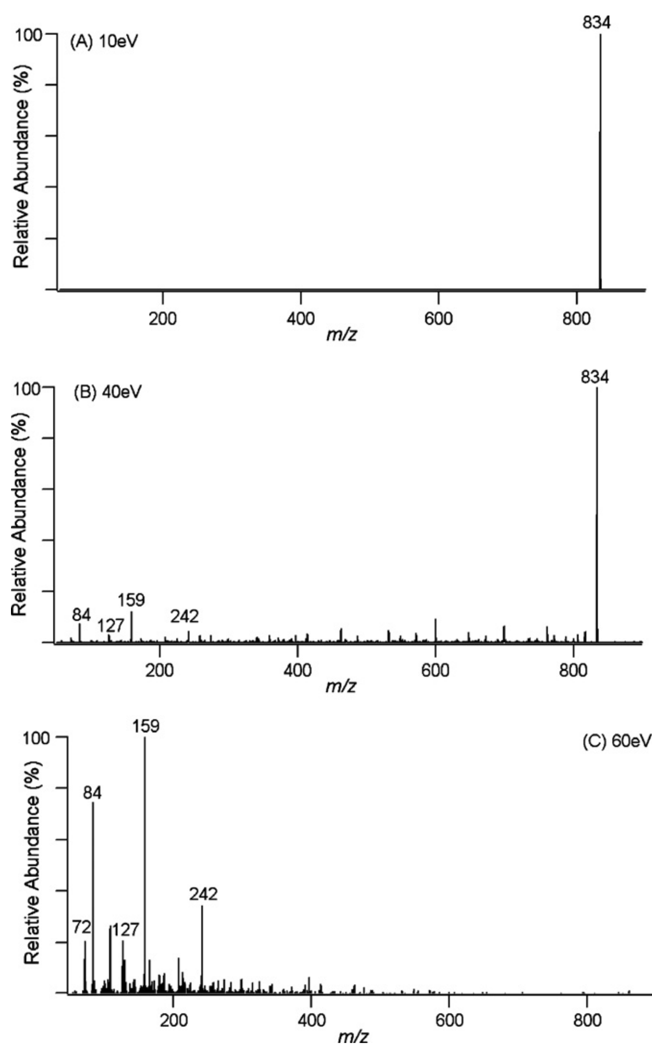

Fig. 4. Product ion spectra of EP-V6 at collision energies of (A) 10 eV, (B) 40 eV and (C) 60 eV.

ence at a CE of 60 eV (panel C). During CE enhancement, numerous subtle peaks were not detected, reflecting the rapid and straightforward dissociation of precursor ions into immonium ions. Among the generated immonium ions, *m*/*z* 159 ($C_{10}H_{11}N_2^+$) is from the double backbone cleavage of the tryptophan residue. The ion at *m*/*z* 127 is the side chain fragment from the Har residue. The less intense product ion at *m*/*z* 72 represents $C_4H_{10}N^+$, which belonged to the valine residue. This corresponds to the ion at *m*/*z* 70, which was derived from the proline residue in the eptifibatide spectrum.

Replacement of proline with valine directly increased the intactness and stability of the loop. This led to a much more complete dissociation of the precursor ion. As a result, EP-V6 had a much "cleaner" spectrum, compared to the intense series of product ions exhibited in the spectra of eptifibatide, in that it lacked the ion series that represents the formation of a linearized sequence. For clarification, one more product ion spectrum was obtained by tuning the collision offset voltage to yield an approximately 10% intensity of the precursor ion peak in the full scan spectrum. Then, 5% intensity of the new peak was set as a threshold to determine the occurrence of product ions. Statistical analysis demonstrated that the extracted number of ions was 7 for EP-V6 and 102 for eptifibatide, providing additional evidence for different fragmentation pathways between the two peptides. Moreover, although both eptifibatide and EP-V6 have the same base peak (*m*/*z* 159), the former only yielded 18% ion intensity compared to the latter. All these facts confirm that the abrupt bond cleavage in the fragmentation of EP-V6 was highly likely promoted by the substitution of proline.

Results mentioned previously suggest that the intactness and stability of the intra-loop structure promote abrupt bond cleavage in tandem mass spectrometry. Also, they suggest that this could be one of the premises for the significant generation of immonium ions. By contract, the presence of proline residues within the intra-loop structure appears to give rise to the sequence linearization, which hampers peptide quantification (Fig. 5).

3.4. Linear peptides

As assumed above, it would be difficult to determine linear peptides using triple quadrupoles, due to the absence of an intra-loop structure and the resulting weak product ions. Surprisingly, quantitative methods have been successfully developed for several linear peptides. It has been reported that the most intense products ions of the 14-amino acid peptide $[M_1]$-PTH(1-14)NH_2 (PTH-parathyroid hormone, M_1-aminoisobutyric acid) are *m*/*z* 58, 110 and 84, corresponding to the residues of aminoisobutyric acid, histidine and lysine, respectively [1]. If utilizing the ion at *m*/*z* 58, the limit of quantitation (LOQ) reached 0.2 ng/mL. Moreover, the product ion used for the quantitative analysis of a 9-amino acid peptide bombesin/GRP receptor antagonist gastrin-releasing peptide (GRP) was the Tpi residue ion (2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid, *m*/*z* 144) [31]. A question that arises from these examples is whether these findings deny the earlier hypothesis concerning the crucial role of intra-loop structures in peptide quantification. Further investigation revealed that the ions used in the above analyses were derived from amino acid residues at either the C- or N-terminal of the linear peptides. For $[M_1]$-PTH(1-14)NH_2, aminoisobutyric acid, histidine and lysine are at the 1, 13 and 14 positions, respectively. For bombesin/GRP receptor antagonist, Tpi is the first amino acid at the C-terminal. Therefore, the underlying mechanism for their high abundance in the mass spectrum is not likely to be the same as for intra-loop immonium ions. It has also been reported that terminally positioned amino acid residues display very intense ions compared to internally positioned residue sites [32]. However, in most cases, the failure to overcome the energy of peptide bond cleavage at the terminals was an obstacle to quantifying linear peptides. A detailed explanation will be expressed elsewhere.

3.5. Bioanalytical assays

Product ion detection in the lower mass range is generally avoided for reasons of low selectivity or high background in MRM analysis [1]. However, Fig. 2 suggests that the immonium ion at *m*/*z* 120 (panel D) may provide more analytical sensitivity than the product ion at *m*/*z* 328 (panel B), which was previously used in MRM as the transition for the determination of a desmopressin analog (i.e., vasopressin) [33]. In the present study, LC/MS/MS assays were developed to assess the usefulness of immonium ions for the detection of peptides with intra-chain disulfide bonds in biological fluids.

Human plasma was spiked with desmopressin at a concentration of 10 ng/mL. The optimized ion transitions of *m*/*z* 535 → 328 and *m*/*z* 535 → 120 were evaluated. As shown in Fig. 6, the peak area at *m*/*z* 120 was almost two times larger than that of *m*/*z* 328 in the extracted ion chromatogram. In addition, the transition of *m*/*z* 535 → 120 had a S/N ratio approximately 5 times higher than for *m*/*z* 328, supporting the high abundance of immonium ions for detection in MRM experiments.

The specificity of immonium ions was demonstrated by an LC/MS/MS assay of EP-V6. Pseudo-MRM transition has been applied to the quantitative analysis of eptifibatide, due to the absence of an

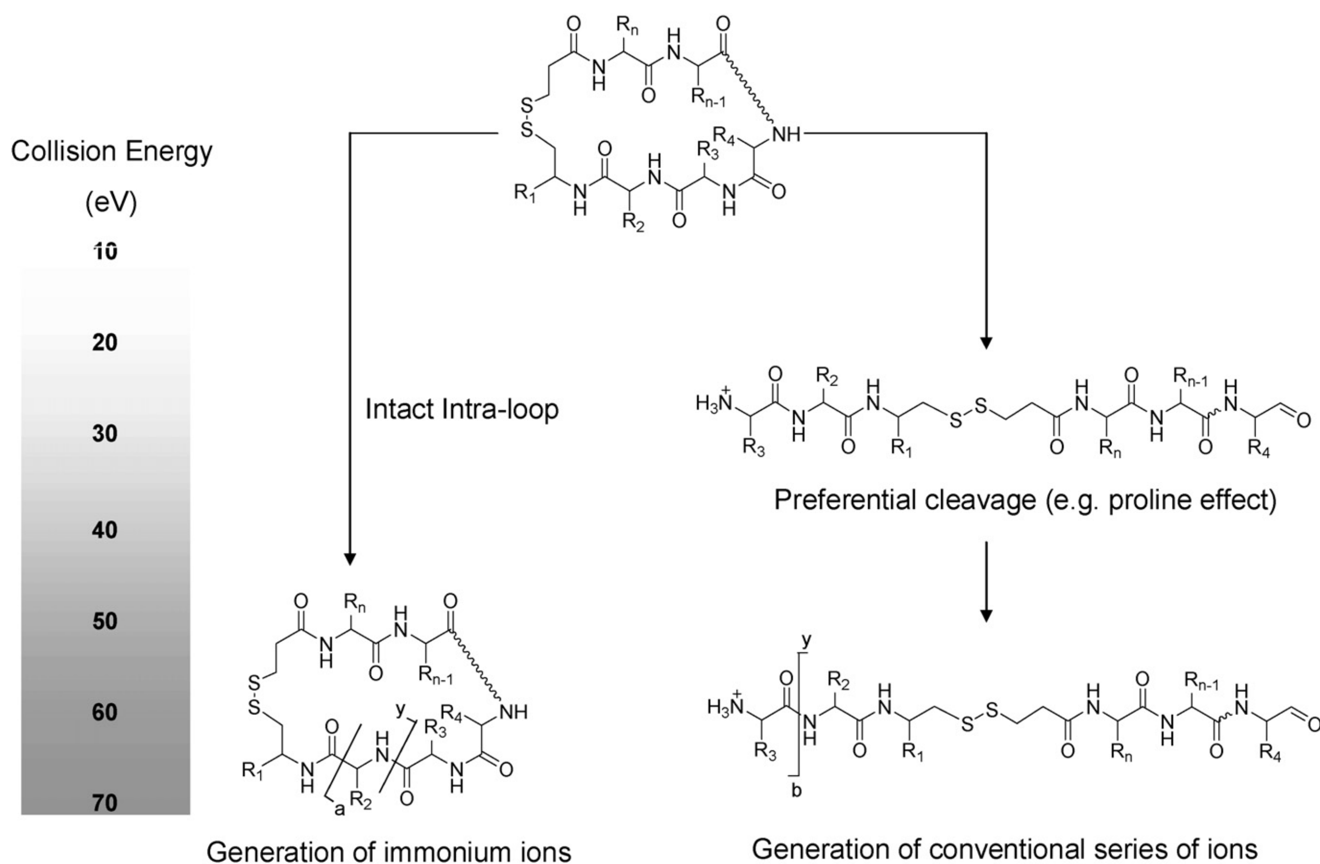

Fig. 5. Proposed collision offset voltage dependent fragmentation pathways for peptides with intra-chain disulfide bonds in triple quadrupole mass spectrometry.

intense product ion [17]. Hence, in the case of EP-V6, a comparison of the pseudo-MRM transition (m/z 834 → 834) and the immonium ion transition (m/z 834 → 159) was performed. An extracted ion chromatogram at 10 ng/mL of EP-V6 showed that both the intensity and S/N of m/z 834 → 159 were higher than those of m/z 834 → 834. This again provides evidence that sensitivity can be improved using immonium ions when abrupt loop cleavage occurs (data not shown). Moreover, excellent linearity, reproducibility and low LOQ were achieved. Notably, in this experiment, no internal standards were used, and injection reproducibility was judged based on duplicate injections. A representative calibration curve, constructed using a weighted linear regression model (weighting factor $1/x^2$), is shown in Fig. S2. The LOQ was 2.00 ng/mL. Linearity was good over three orders of magnitude (from 2 to 250 ng/mL; calibration curve: $y = 76.9x + 83.8$; correlation coefficient: $r^2 = 0.9984$), and the accuracy of back-calculated concentrations was within 85.0–108.0%.

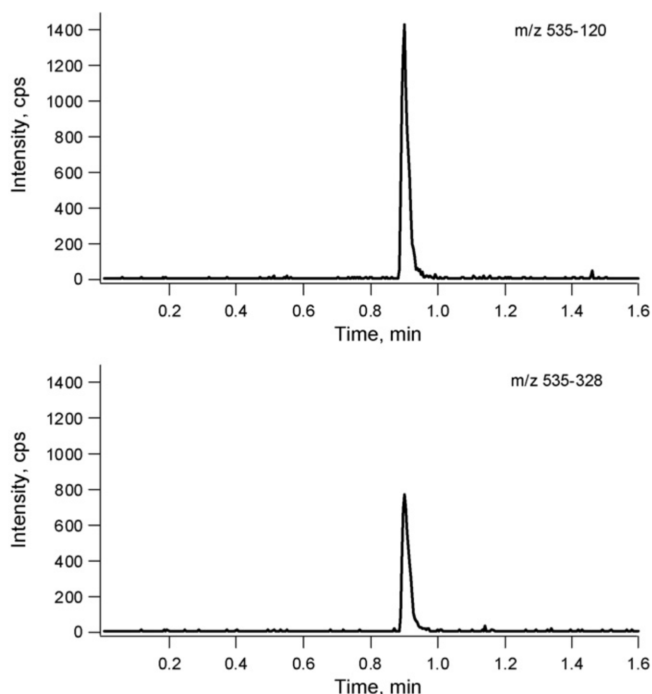

Fig. 6. Chromatograms of human plasma spiked with 10 ng/mL desmopressin in MRM transitions of m/z 535 → 120 (A) and m/z 535 → 328 (B).

4. Conclusion

It has been suggested that the formation of fewer fragment ions with higher relative intensities has a direct and positive impact on the overall sensitivity of a quantitative assay [30]. Consequently, triple quadrupoles are usually not considered as the instrument of choice for peptide determination, due to their multi-step fragmentations and generation of multiple weak peaks. However, it has been discovered that some peptides with intra-chain disulfide bonds are sensitive to this type of mass detection. By employing several peptide analytes, this work demonstrates that the intra-loop structure in peptides can increase fragmentation efficiency and raise the number of immonium product ions produced, provided that there is no peptide bond susceptible to the cleavage (e.g., proline). With these conditions, the quantitative analysis of peptides with intra-loops in biological fluids can be achieved using immonium product ions to develop simple and sensitive LC/MS/MS assays. Finally, the strategy described here might also be appli-

cable to other small cyclic peptides, as well as large peptides (MW > 1000). Future work will focus on the determination and quantification of these peptides by triple quadrupole mass spectrometry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2009.08.008.

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