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An investigation into fragmentation of hEGF in triple quadrupole mass spectrometry and its quantitative application to human plasma

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ABSTRACT

A growing number of peptides are being used today as biomarkers and therapeutic drugs. Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS), in providing a highly specific and reliable technique, is likely to have a substantial impact on peptide quantification. However, most of the applications developed in research laboratories have relied on capillary liquid chromatography, nanospray interfaces, and ion trap or other types of mass spectrometers, which are not commonly found in preclinical and clinical laboratories. Triple quadrupole mass spectrometers are not often considered for quantitative analysis of peptides, despite the fact that they are currently an increasingly common tool in preclinical and clinical laboratories. However, sensitive peptide analyses have been reported, as in the demonstration of structurally distinctive immonium ions that were formed in abundance. Thus, we have recently proposed that use of immonium ions might provide a potential pathway for the quantitative analysis of oligo-peptides with intra-chain disulfide bonds. In the present report, the capability of these ions in LC/MS/MS toward peptide determination was further explored using a polypeptide, human epidermal growth factor (hEGF). hEGF is much larger and more complex than the oligo-peptides previously investigated in our lab. Both the experiments and theoretical calculations were carried out. The results indicate that polypeptides with intra-chain disulfide bonds can increase fragmentation efficiency and raise the number of immonium product ions produced, on the condition that at least one of their intra-loops is sensitive to abrupt bond cleavage. In addition, the utilization of immonium product ions in LC/MS/MS was demonstrated for the determination of hEGF in human plasma. Good linearity and accuracy were achieved.

1. Introduction

An increasing number of peptides are being used today as biomarkers (or their quantitative target) and therapeutic drugs in preclinical and clinical laboratories [1,2]. Presently, peptide quantification is often based on bioassays, such as enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays (RIAs). These methods rely on the specificity of antibodies for capture and detection. Thus, their performance is largely subject to the antibodies obtained and biological interference. Because of this, liquid chromatography coupled with tandem mass spectrometry, in providing a highly specific and reliable technique, is likely to have a substantial impact on peptide quantification in laboratories. However, most of their applications developed in research laboratories have relied on capillary liquid chromatography, nanospray interfaces, and ion trap or other types of mass spectrometers, which are not commonly found in preclinical and clinical laboratories [3]. Therefore, current methods using these techniques are usually non-generic in detection and quantification.

Triple quadrupole mass spectrometers are currently an increasingly common tool in preclinical and clinical laboratories. However, they are not often considered for quantitative analysis of peptides [4]. With this instrumentation, collision cascades or multiple fragmentations tend to generate multiple peaks that have weak intensities during the fragmentation process of peptides [4]. This leads to a loss in detection sensitivity. However, sensitive peptide analyses have been reported, as in the demonstration of structurally distinctive immonium ions that were formed in abundance [5]. This was achieved via the combination of a- and y-type cleavages during secondary fragmentation [6]. To date, immonium ions have been largely overlooked due to the dominance of ion trap instruments in the field of mass spectrometry-based proteomics [7]. However, they are visible in triple quadrupole mass spectrometers. Several reports have used this type of ions in LC/MS/MS quantification of

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peptides and larger biological molecules [5,8,9]. Murao et al. have developed a simple and sensitive method for the determination of hepcidin-25 using the immonium product ion of phenylalanine residue [5]. Salek et al. have used the immonium ions of monoiodo- and diiodo-tyrosine as highly specific marker ions for tyrosine iodination [9]. 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) [8]. Nevertheless, the quantitative capability of immonium ions using triple quadrupoles has not yet been fully recognized and extensively elucidated. Recently, we have suggested that the use of immonium ions might provide a potential pathway for the quantitative analysis of oligo-peptides with intra-chain disulfide bonds [10]. To further explore the capability of these ions in LC/MS/MS toward peptide determination, a polypeptide human epidermal growth factor was investigated here.

hEGF consists of 53 amino acids, and the molecule is stabilized by three intra-disulfide bonds [11]. It plays an important role in the regulation of cell growth and proliferation. However, it also exhibits 'tumor promotion' [12,13]. These observations have raised doubts on the clinical safety of hEGF. A variety of studies are being conducted with the goal of accessing hEGF indication and safety implication [14–16]. Thus, a robust, specific and sensitive analytical assay for its detection and quantification is eagerly anticipated in preclinical and clinical laboratories. hEGF is four-fold larger and structurally more complex than the oligo-peptides previously investigated in our lab [10]. Therefore, more experiments are required to evaluate whether the earlier reported gas-phase fragmentation patterns of oligo-peptides can be observed for hEGF using triple quadrupoles. In addition to experimental evidence, theoretical model calculations can also provide useful insight into the fragmentation process.

In this report, the potential of immonium ions in LC/MS/MS was explored using hEGF 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. The usefulness of intra-loop structures in peptide fragmentation efficiency and the number of immonium product ions produced was elucidated as well. Furthermore, quantum chemical calculations [17] of a simple model were carried out to evaluate the proposed fragmentation mechanism. Finally, an investigation into the quantitative eligibility of using immonium product ions in LC/MS/MS was performed for hEGF in human plasma.

2. Experimental

2.1. Chemicals and reagents

hEGF was purchased from Sinobio Biotec (Shanghai, China). Acetonitrile (ACN) and formic acid were of 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). Drug free EDTA human plasma was provided by Jiangsu blood center (Nanjing, China).

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 0.3 ml/min was applied in the following manner (duration in parentheses): B 5%(0 min) → 5%(0.5 min) → 95%(8.0 min) → 95% (9.0 min) → 5% (9.5 min) → 5% (10.0 min). The injection volume was 10 μL.

The mass spectrometer was interfaced with an electrospray ion source and operated in the positive multiple reaction monitoring (MRM) mode. Q1 and Q3 were both set at unit resolution. The flow of the desolvation gas 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 4.0 kV and 55 V, respectively. Data were collected and processed using MassLynx 4.1 from Micromass–Waters (Manchester, UK). The collision energy was monitored in steps of 5 eV from 0 to 60 eV, with all other settings held constant. Two ranges of energy (i.e., 0–30 eV and >30 eV) were introduced to distinguish collision conditions that led to different fragmentation patterns [10,18]. 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 [19–22].

The ion transition used for hEGF quantification was m/z 1037 → 136. 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 and infusion solutions

Peptide was accurately weighted. Stock solution was prepared at 1 mg/ml by dissolving peptide into the working solvent ACN:water (50:50, v:v) containing 0.1% formic acid. It was stored in a brown glass tube, which protected it from light, at −20 °C. The stock solution was diluted to 1000 ng/ml for the infusion solution. It was stored at 4 °C.

2.4. Preparation and pretreatment of plasma samples

hEGF calibration standards were prepared by diluting the stock solution with human plasma on ice. Concentrations of the calibration standards were 10, 25, 50, 100, 250, 500 and 1000 ng/ml (1.60–160 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.

2.5. Computational methods

The molecular model was build upon the X-ray crystal structure of a hEGF molecule in the Protein Data Bank (code 1JL9) [23]. Bond length was calculated to determine the order of bond strength in the molecule. To reduce the computational cost, side chains of the molecule were truncated and replaced with hydrogen atoms. The geometry was then optimized using Becke's three-parameter hybrid functional combined with Lee–Yang–Parr correlation (B3LYP) at B3LYP/6-31G*//B3LYP/6-31G* level [24,25]. All quantum chemical calculations were done using Gaussian 03 [26].

3. Results and discussion

3.1. Triple quadrupole mass spectra of hEGF

In the positive ionization mode, peptides normally have a number of suitable sites for protonation such as all of the backbone

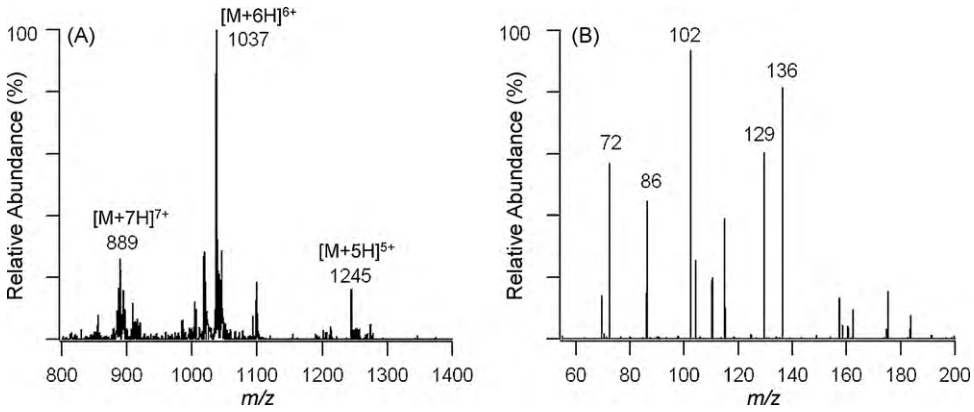


Fig. 1. Full scan spectrum of hEGF (A) and product ion spectrum of its six-fold charged molecular ion (B).

amide nitrogen atoms could be protonated theoretically, as well as certain basic amino acid residues. Thus, multiply charged molecular ions are characteristic for ESI-MS analysis of peptides. In this case, three main multiply charged ions of hEGF are shown in the full scan spectrum (Fig. 1A). The base peak at m/z 1037 corresponds to its six-fold charged precursor ion. The ions at m/z 889 and m/z 1245 are the seven-fold and five-fold charged ions. Other multiply charged ions were not found in the spectra (data not shown). The subsequent tandem mass spectrometry (MS/MS) study of hEGF was performed by collision-induced dissociation (CID) of these molecular multiply charged ions. The ion m/z 1037 was first considered as the choice of precursor ion because of its abundance. At this point, the limit of detection of the method could possibly be lowered by using the most abundant precursor ion. It is also noteworthy that the distribution of charged states would likely change under different solvent conditions [27,28]. Correspondently, the alteration can influence the choice of precursor ion in the subsequent monitoring. For example, low pH tends to create a charge state distribution centering on more highly charged ions in the lower m/z region of the spectrum due to the accessibility of more charges [29].

In MS/MS analysis, collision energy (CE) was monitored in steps of 5 eV from 0 to 60 eV, with all other settings held constant. With the application of CE in stepwise increments, numerous fragments spanning the middle mass range of spectra were shown at low collision energies. However, none of these was strong enough for quantification. After higher energy (i.e., >30 eV) was introduced, product ions in a lower mass range (e.g., m/z 72, 86, 102, 129 and 136) were observed in the product ion spectrum, for example, under CE at 45 eV (Fig. 1B). Their peak intensity was high compared with the ions in the medium mass range detected under lower CE conditions. Afterward, no significant changes in ion composition of this mass range were observed in the spectra. To illustrate their prevalence in the product ion spectra of hEGF under higher energy collisions, the fragmentation efficiency curve of ion m/z 86 was depicted as an example (Fig. 2). In the plot, the percentage of this ion compared to the total ion count increases with CE, achieving its apex and most dominant presence of ~5.5% at a CE of 45 eV. Further evaluation of generated product ions in the low-mass range, following high collision offset voltages, predominantly showed immonium ions. This type of ions, with a mass 27u lower than that of the corresponding amino acid residue, has been suggested as a good indicator of the presence or absence of the particular amino acid in the peptide sequence [6,30]. In this case, the most intense immonium ions were at m/z 102 and m/z 136, which correspond to glutamic acid residue and tyrosine residue. Other immonium ions were likely derived from valine (m/z 72), isoleucine/leucine (m/z 86) and arginine (m/z 129).

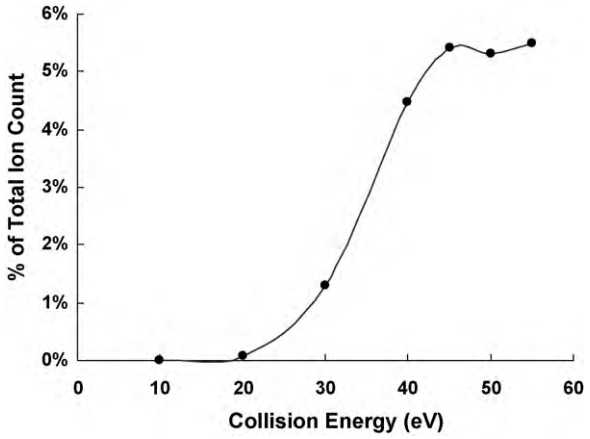


Fig. 2. Fragmentation efficiency as a function of collision energy for the transition of m/z 1037 → 86.

3.2. Immonium ions generation

As mentioned earlier, the employment of immonium ions in LC/MS/MS quantitative assays has been growing. However, the underlying mechanism for immonium ions generation and the prospects for their future applications have been rarely discussed. Two parameters will determine their quantitative capability in LC/MS/MS [9], (i) the efficiency of immonium ion formation, which is residue-specific, and (ii) the 'uniqueness' of the immonium ion mass with respect to common fragment ions of peptides and to typical background ions of triple quadrupoles.

Regarding to the abundance of immonium ions generated, we have recently proposed that intense generation of immonium ions could be achieved by abrupt bond cleavage from the intra-loop structure of peptides, given that there is no peptide bond susceptible to the cleavage (e.g., proline) [10]. In the case of hEGF, it has three intra-disulfide loops. One is CVVGYIGERC (Fig. 3), which consists of 10 amino acids. As illustrated in the sequence of CVVGYIGERC, most residues within this loop (except glycine; due to mass cutoff in this experiment) had their derived immonium ions in the spectrum. Taken together with their abundance as described previously, these facts suggest that loop opening was probably achieved by a rapid and straightforward dissociation into low-mass fragments. By contrast, some desired immonium ions corresponding to the residues within other two loops (e.g., D m/z 88, S m/z 60 and K m/z 101) were not present. Specifically, the amide bond adjacent to proline within the loop of CPLSHDGYCLHDGVC is a predetermined cleavage point, which gives rise to the sequence linearization prior to fragmentation at low CE [30–32]. Further fragmentation of the linearized peptide can occur; however, formation of immonium ions seems

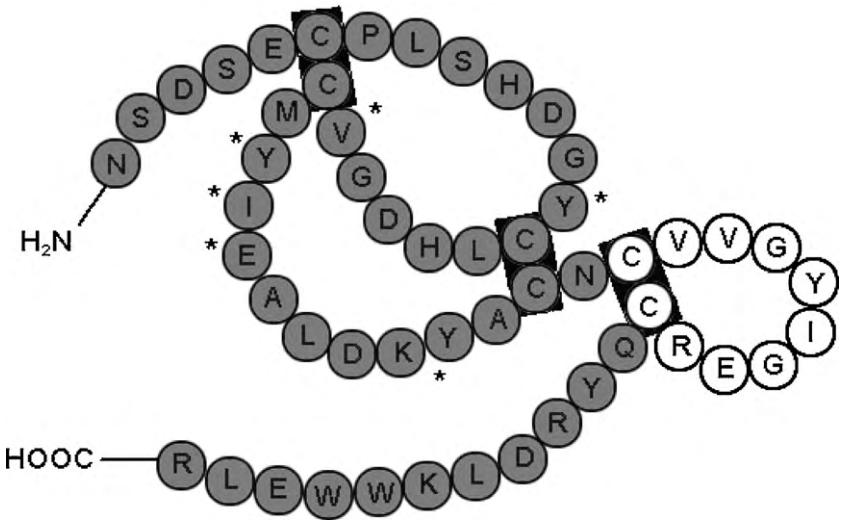


Fig. 3. Primary sequence of hEGF. Some residues within the shadowed peptide loops (asterisk) may contribute to the abundance of immonium ions.

less probable [10,33]. For the largest loop (i.e., CLHDGVCMYIEALDKYAC), the relative incompleteness of dissociation were probably caused by the larger number of loop members, which could directly decrease the intactness and stability of ring structure [34].

As to the second parameter (i.e., the 'uniqueness' of immonium ions), there was an increasing evidence that they were highly specific ions in detection and quantification of peptides [7,9,33]. In this study, the results of blank plasma indicated that the ion transition detected was free of interference arising from matrix components (see Section 3.4).

3.3. Simple quantum chemical estimation

Quantum chemical calculation was also carried out in this study to provide a theoretical insight. To simplify the calculations, only the loop with the largest number of residues having their derived immonium ions in the product ion spectra was investigated. Thus the sequence of CVVGYIGERC with a disulfide bond was separated out for calculation (Fig. 4A). Its corresponding linear peptide was also investigated to illustrate the impact of intra-loop structure on bond strength distribution (Fig. 4B). In the linear structure, the disulfide bond was broken and hydrogen atoms were added to form thiol groups, –SH. After the quantum chemical calculations, the resulted bond lengths of amide bonds in the optimized structures are shown in Fig. 4C. Almost all the intra-loop amide bonds had their lengths in the range of 1.35 and 1.36 Å, whereas less consistency was observed in the linear structure. The Cartesian coordinates of all the atoms are listed in Supplemental Material.

The results of quantum chemical calculations also provided increased support for the above mechanism of the intra-loop structure. The relative uniformity of bond stress could effectively prevent preferential cleavage of the amide bond, disruption of the stable ring structure and linearization of the peptide sequence. This intactness and stability of the loop can finally lead to abrupt bond cleavage, as well as immonium ion generation in abundance. On the other side, the larger variation in bond length and strength of the linear peptide suggested that its amide bond dissociation may be more subject to the bond position in structure. It has been reported that terminally positioned amino acid residues may display very intense ions compared to internally positioned residue sites [33]. Even though immonium ions can be derived from amino acid residues at either the C- or N-terminal of the

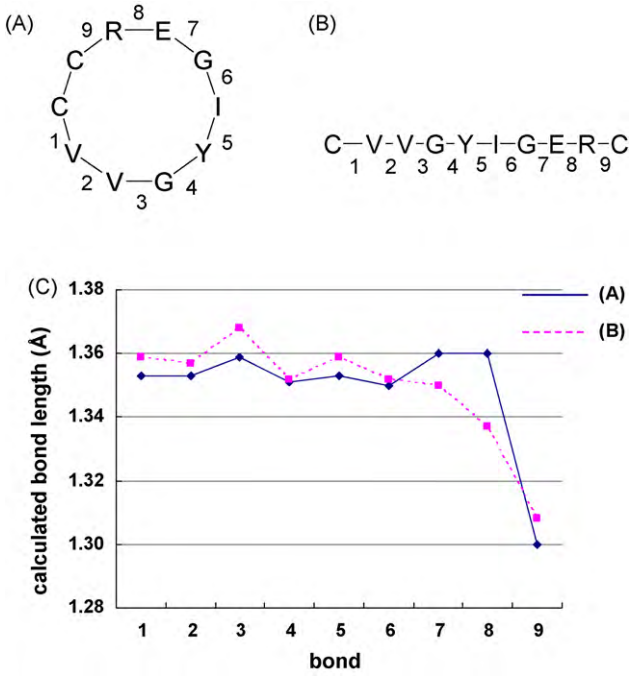


Fig. 4. Intra-disulfide loop of CVVGYIGERC (A) and its corresponding linear structure (B) with bond labels. The bond lengths (Å) calculated for their optimized structures at B3LYP/6-31G*//B3LYP/6-31G* level of theory are also shown (C).

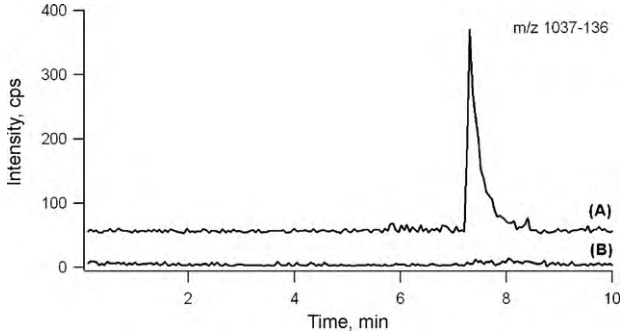
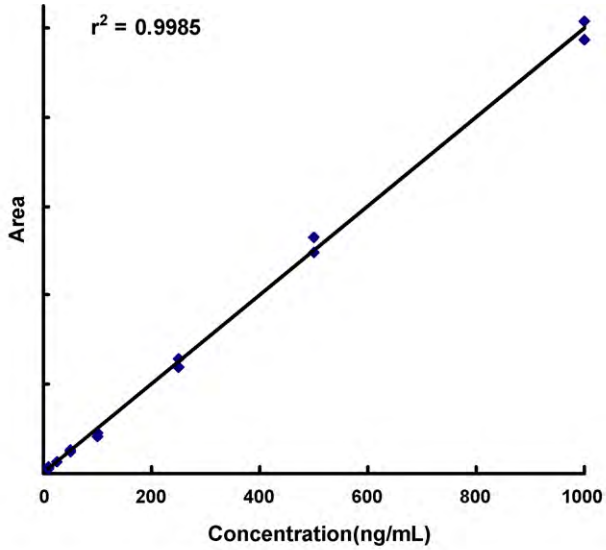


Fig. 5. Chromatograms of human plasma spiked with 25 ng/ml hEGF (A) and blank plasma (B) in MRM transition of m/z 1037 → 136. Top trace is offset on the y-axis for clarity.



hEGF	Standards (ng/mL)						
	10.0	25.0	50.0	100	250	500	1000
Accuracy	*154.4	99.4	102.0	91.0	103.1	106.3	101.5
	109.4	110.5	102.6	85.2	95.6	99.0	97.4

* *Deactived*

Fig. 6. Calibration curve (10 ng/ml to 1000 ng/nL) for hEGF standards prepared in human plasma. All injections were performed in duplicate. Accuracy of the standards for the determination of hEGF in human plasma was also listed.

linear peptides/linearized peptide sequences and have been used for quantification as earlier mentioned [5,8,33,35], the underlying mechanism for their high abundance in mass spectrum is not likely to be the same as for intra-loop immonium ions. It is worth pointing out that the model employed here might be the simplest one for quantum chemical calculations. The interaction of intra-loop with chemical environment and side chains were not taken into account. Both of them have been initially truncated to minimize theoretical challenge and computational expense. More complex models considering these effects will be explored elsewhere.

3.4. LC/MS/MS

In the present study, a LC/MS/MS assay was developed to assess the usefulness of immonium ions for the detection of polypeptide hEGF in biological fluids. After optimization, immonium product ion at m/z 136 provided the best sensitivity. Therefore, the ion transition of m/z 1037 → 136 was employed for hEGF detection. Human plasma was spiked with hEGF at a concentration of 25 ng/ml. A comparison of the pseudo-MRM transition (m/z 1037 → 1037) and the immonium ion transition (m/z 1037 → 136) was performed. In a pseudo-MRM, the analyte was detected by means of a protonated molecule [36]. A number of studies have applied pseudo-MRM chromatography to the quantitative analysis of peptide, due to the absence of an intense product ion [26,37]. As a result, good chromatograms of human plasma spiked with hEGF can be obtained using the immonium ion transition (Fig. 5A). Both the intensity and signal to noise (S/N) of m/z 1037 → 136 were higher than those of 1037 → 1037 (data not shown). Blank plasma was also analyzed to investigate whether there was any interference from endogenous substances with hEGF. Consequently, no significant interfering peak from the plasma was found at the retention time in the transition of m/z 1037 → 136 (Fig. 5B). A representative calibration curve, constructed using a weighted linear regression model (weighting factor $1/x^2$), is shown in Fig. 6. The limit of quantification (LOQ) was 10 ng/ml. Linearity was good over three orders of magnitude (from 10 to 1000 ng/ml; correlation coefficient: $r^2 = 0.9985$), and the accuracy of back-calculated concentrations was within 85.2–109.4%.

In general, product ion detection in the lower mass range is avoided for reasons of low selectivity or high background in MRM analysis [5]. However, this study provides evidence that sensitivity can be improved using immonium ions when abrupt loop cleavage occurs. Moreover, good linearity and accuracy were achieved. Notably, the normal levels of hEGF in human plasma and urine were around 10 ng/ml [38], fairly approaching to the LOQ in this study. Thus there is possibility that the LC/MS/MS method developed here may not be adequate for real preclinical and clinical samples. To achieve better sensitivity and avoid information missing or largely distorted, appropriate separation and concentration strategy may be required in future experiments. Sample pretreatments such as solid phase extraction [39] and online extraction [40] can help to achieve this goal. Their enrichment effects could improve the sensitivity 1–3 orders of magnitude [39]. In this way, the concentration of hEGF in most biological fluids would be likely traced. While the sample preparation is not the focus of this report, a detailed discussion will be given elsewhere.

4. Conclusion

Both the experimental evidence and theoretical calculation of hEGF provide evidence that polypeptides with intra-chain disulfide bonds can increase fragmentation efficiency and raise the number of immonium product ions produced, given that at least one of their intra-loops is sensitive to the abrupt bond cleavage. The relative abundance of immonium product ion can facilitate its MRM monitoring in LC/MS/MS using a triple quadrupole mass spectrometer. This successful detection is likely to promote the application of LC/MS/MS to peptide quantification in preclinical and clinical laboratories. However, these prospects require further experiments

to provide more evidence concerning the crucial role of intra-loop structures in peptide quantification. In addition, the present quantum calculations can be improved by including more effects of interactions. Finally, in response to the potential of using this strategy to identify and quantify peptides or modified peptides in trypsin digested mixture, appropriate component separation or high resolution mass spectrometers may be involved to distinguish the target ions from dramatically increased fragment ions and background ions after digestion [7,41].

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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.2010.06.010.

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