UDK 577.1:543.51
Review
Received: 3. 09. 2010.
Accepted: 20. 10. 2010.

SEQUENCE DETERMINATION OF PHARMACEUTICAL PEPTIDES BY MALDI-TOF TANDEM MASS SPECTROMETRY

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Summary

Although the peptide ion mechanisms of pre-dissociation, dissociation and post-dissociation have been well-studied over the past fifteen years, their practical application still has to be implemented into modern mass spectrometry-driven proteomics and bioanalysis. Unambiguous peptide sequence determination by mass spectrometry relies on the idea that only one continuous series of ions in mass spectrum can assure full sequence determination and meets the requirements of peptide analysis quality. This set of rules defined for the peptide analysis by tandem mass spectrometry would generally improve an overall reliability and data accuracy. Based on the process mechanisms of gas-phase peptide bond dissociation, a relatively small and large model peptides are unambiguously analyzed (bivalirudin and exenatide) showing that derivatization concepts of the C- or N-terminus derivatization (SPITC and Lys-tag) can be avoided.

Keywords: MALDI-TOF/TOF; sequence determination; pharmaceutical peptide; bivalirudin; exenatide

INTRODUCTION

Unambiguous structural and sub-structural sequencing of a protein or a peptide should be desirable final result of tandem mass spectrometry analysis. Prerequisites for unambiguous protein/peptide structural and sub-structural analysis are sufficient mass accuracy and sensitivity, and continuous series of signals where each signal represents particular amino acid or modified amino acid (e.g. modified by post-translational modification) [1]. Apart from several known obstacles in MS analysis of peptides, e.g. hampered discrimination

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between leucine and isoleucine (additional high-collision MS/MS experiment is required with production of w and d satellite ions that can enable leucine and isoleucine to be differentiated) [2], and between lysine and glutamine (due to small mass difference of *m*/*z* 0.0364) [3], unambiguous peptide sequence determination should theoretically be a standard procedure performed by tandem mass spectrometry [4-7]. .Although just a couple of MS/MS detected fragments (sequence tag data processing) [8] are adequate for positive peptide identification in the database, the result of the analysis is always given with a certain statistical probability [9]. Calculated probability can be high, or more probable low or even uncertain if post-translational modifications are present, [10-12], but when *de novo* sequencing is required, sequence tag data processing is not always suitable for unknown peptide structural determination [13,14].

Modern high-throughput proteomics is designed to work with reasonable number of mishit amino acids in the peptide in opposite to pharmaceutical bioanalysis of peptides where unambiguous sequence analysis rule must be followed by either mass spectrometry and/or Edman sequencing (preferentially with spectroscopic control evaluation, e.g. NMR) [15,16]. The mobile proton model, as well as localized proton frameworks can be explored for both types of analysis (proteomics and biopharmaceutical analysis) leading ultimately to infallible and unambiguous tandem mass spectrometry peptide sequence analysis [1,4,17]. Synthesized peptides [18] with or without prosthetic groups or with chemically modified amino acids or termini provide almost ideal experimental framework for the mobile proton model application, especially for the chemical structures (e.g. cyclic peptides [4,19,20] and some post-translational [21] or chemical modifications [22]), where the model deviated from predicted fragmentation pattern (i.e. the proton affinity of cyclic peptides is much smaller than that of linear peptides).

Synthetic pharmaceutical peptides are produced mainly by organic synthesis (either by solid phase or in-solution), and sometimes by bio-synthesis that might be advanced by final chemical peptide modification(s) [23,24]. Biopharmaceuticals grow in size and become more complex as the peptide chemistry constantly improves their production methodology and scales up manufacturing. Final quality control was developed in parallel with the progress of peptide synthesis science, and it includes analytical evaluation of raw materials and finished products [16]. The assessment of structural integrity and specified quality of the peptide is critical during the developmental stages of a product and later formulation/stability studies [25]. Identity evaluation of a given peptide mostly utilizes analytical techniques such as HPLC, NMR and mass spectrometry. There is no fast and simple analytical measurement that can assure unambiguous amino

acid sequence determination, especially when prosthetic groups (e.g. phosphates, lipids, carbohydrates) are incorporated in the peptide structure [26]. However, NMR would be the best choice, but in many cases the number of samples, sample quantity, duration of the analysis, less complex data interpretation and high-throughput screening of formulation/stability samples are limiting factors [27,28]. Therefore, mass spectrometry as a single technique or hyphenated by liquid chromatography very often takes place in the final assessment of (bio) synthesized molecule/macromolecule [29, 30].

ESI-TOF or MALDI-TOF MS, and consequently MS/MS measurements are frequently used as fast, accurate and reliable analysis tool, which has a drawback when analyzing isobaric ions (the ions of the same nominal mass but different chemical structures). The isobaric chiral ions and some other specific type of isomer structures cannot be differentiated by routine mass spectrometry applications [31,32] (i.e. different oligosaccharide linkages [33], or deamidated ions [34] overlapping with C13 isotope envelopes or even simpler isoleucine/leucine differentiation). Nevertheless, this problem might be overcome by hyphenation of mass spectrometry to liquid chromatography or by usage of specific mass spectrometry features (e.g. high energy collision-induced dissociation, ion mobility or high resolution MS with R > 100000).

Sub-structural unambiguous analysis by tandem mass spectrometry includes analysis of the biopolymer in one ion series in the ladder sequence covered 100% with x-, y-, z- or a-, b- c-series ions. Sometimes, it is simply not feasible because cleavages of some amino acids in particular series are more preferred than the others, and thus structural elucidation of peptide sequence must be performed by joining together mass spectrum data collected from two or more ion series (usually, after collision-induced dissociation y- and b-series ions are more abundant than neutral loss sequence ions: $-H_2O$, $-NH_3$, possibly $-H_3PO4$; or $b + H_2O$, or satellite forms d, v or w, or internal fragmentation or rearrangement ions) [35]. The product ion mass spectrum can be either incomplete or overfilled with ions (chemical noise) in important segments hampering sequence analysis and aggravating final sequence assessment. The mass spectra of prosthetic group-containing peptides might become more incomplete or inexplicable in comparison to their unaltered peptide counterparts. As a consequence, mass spectra must be simplified, more populated with desired series of ions and unaffected by prosthetic group interferences.

In many cases of unambiguous mass spectrum analysis, simple and fast derivatization or enzymatic reaction can modify peptide in such a way as to become more amenable to unambiguous amino acid sequence or prosthetic group mass

spectrometry analysis. Specific and selective enzymes, e.g. trypsin, Lys- or Arg-C can cut down the peptide into smaller pieces, leaving the polar and basic terminal group at C-terminus, which would localize proton consequently enhancing y-series ions intensity. It is worth to know prior to analysis that in ionization source, the MS ionization of Lys-terminated fragments might be suppressed by the presence of Arg-terminated fragments, and in MS/MS spectra, Arg-terminated peptides give better ion yield of almost exclusively y-series ions. Arginine has the highest proton affinity amongst all amino acids, and proton localized on arginine can be therefore mobilized by the highest activation barrier [36].

To improve the MS potential of Lys-terminated fragments *versus* Arg-terminated fragments, lysines can be derivatized by 2-methoxy-4,5-dihydro-1*H*-imidazole (Lys-tag) or *O*-methylisourea. Lys-tag derivatization reaction increases the ionization efficiency and simplifies tandem mass spectra at the same time. The same effect, but inversed termini polarity can be achieved by derivatization of N-terminus amine with SPITC (4-sulfophenyl-isothiocyanate), strong acid that provides proton donation to the backbone amide bonds making MS/MS spectra simpler and more populated with y-ions [13,37].

Oxidated cysteines and disulfide bonds also interfere with MS/MS analysis in positive ion mode by intercepting the sequence ladder exactly up to the disulfide bond formation site [38]. Acrylation, methylation or carboxymethylation derivatization reactions are widely used in proteomics in preventing intra-molecular and inter-molecular disulfide bond formation between cysteine residues [39]. The same approach with MS and MS/MS analysis compared before and after cysteine derivatization can determine position of disulfide bonds in the peptide.

Tandem mass spectrometry analysis of peptide–containing prosthetic groups as phosphates or carbohydrates with number of free hydroxyl groups diminish ionization efficiency in positive ion mode and reduce structural data information collection in negative ion mode [40,41].

In the present paper, we chose model peptides bivalirudin (Angiomax®) and exenatide (Byetta®) to illustrate the unambiguous analysis by MALDI-TOF MS/MS. Bivalirudin is an icosa-peptide produced by solid phase synthesis that directly inhibits thrombin by binding simultaneously to its active catalytic site and its substrate recognition site [42]. Peptide does not contain any modification of C- or N-terminus and prosthetic groups are not part of Bivalirudin molecule.

Exenatide is approved for the treatment of diabetes mellitus type 2, and Enfuvertide is an anti-viral drug used in combination therapy for the treatment of HIV-1 infection [43]. The C-terminus of named pharmaceutical peptide is amidated.

MATERIALS AND METHODS

Peptides. Peptide samples investigated in this study: Bivaluridin and exenatide were purchased from Chengdu Kaijie Bio-Phamaceuticals Co., Ltd. (China). The stock solution concentrations were 0.1 mg/mL.

Matrix. For both MALDI–TOF MS and MS/MS analysis, alpha-cyanohydroxycinnamic acid (CHCA) matrix solution (concentration of 5 mg/ml, prepared in 50% acetonitrile) was used. Matrix was purchased from Sigma–Aldrich (St. Louis, USA). For sample analysis, dried purified samples (both derivatized and non-derivatized) were diluted in 5 μl of CHCA matrix solution and 1 μl of mixture was spotted onto MALDI plate and allowed to dry.

Samples Preparations

Bivalirudin. 10 μl of sample stock solution was desalted using Zip-Tip C4 pipette tips and dried.

MALDI-TOF/TOF. A model 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems Inc., Foster City, CA, USA) equipped with a 200 Hz, 355 nm Nd:YAG laser was used for direct profiling and MS/MS fragmentation study. Acquisitions were performed in positive ion reflectron mode. Instrument parameters were set using the 4000 Series Explorer software version (V 3.5.3, Applied Biosystems Inc., Foster City, CA, USA). Mass spectra were obtained by averaging 1000 laser shots covering mass range *m/z* 9 to 4500. MS/MS was achieved by 1 or 2 kV collision induced dissociation (CID) in positive ion mode.

RESULTS AND DISCUSSION

Unambiguous MS/MS analysis of intact bivalirudin peptide.

Intensity of peptide fragment ions in CID (collision-induced dissociation) MS/MS spectra is uneven and sometimes unpredictable. Tryptic peptides analyzed by tandem mass spectrometry have charge distribution that facilitates the MS/MS unambiguous amino acid sequence determination, but most of the synthesized peptides are not arginine- or lysine-terminated. Besides amino acid composition, size of the peptide is also important factor in unambiguous peptide analysis. Modern time-of-flight (TOF) mass spectrometers can easily dissociate the peptide having the size of *m/z* 5000, but a general rule derived from these experiments might be defined as the bigger the peptide is, the bigger will be the gap of particular series of ions in mass spectrum. Bivalirudin is a relatively small-sized peptide (monoisotopic protonated *m/z* 2180) with amino acid com-

position that should be easily fragmented (NH_2-FPRPGGGGNGDFEEIPEEYL-COOH). This assumption is based on the fact that 3 prolines and 4 aspartic acids are present in the peptide chain. The most intense ions in the MS/MS spectrum should be produced by fragmentation on the amino-terminal side of proline. Additionally, peptides that contain aspartic acid prefer to fragment at the carboxyl-terminal adjacent amide bond. In both cases, y-series of ions should be expected and predominant in tandem mass spectrum but this would not guarantee 100% coverage of the y-series ions. Tandem mass spectrometry experiment and mass spectrum showed on Figure 1 proves that theoretical predictions of peptide sequencing are useful, but complete sequence of specific peptide cannot be anticipated. Unambiguous amino acid sequence was determined by b- and alternatively a-series of ions where proline or aspartic acid negatively affected the

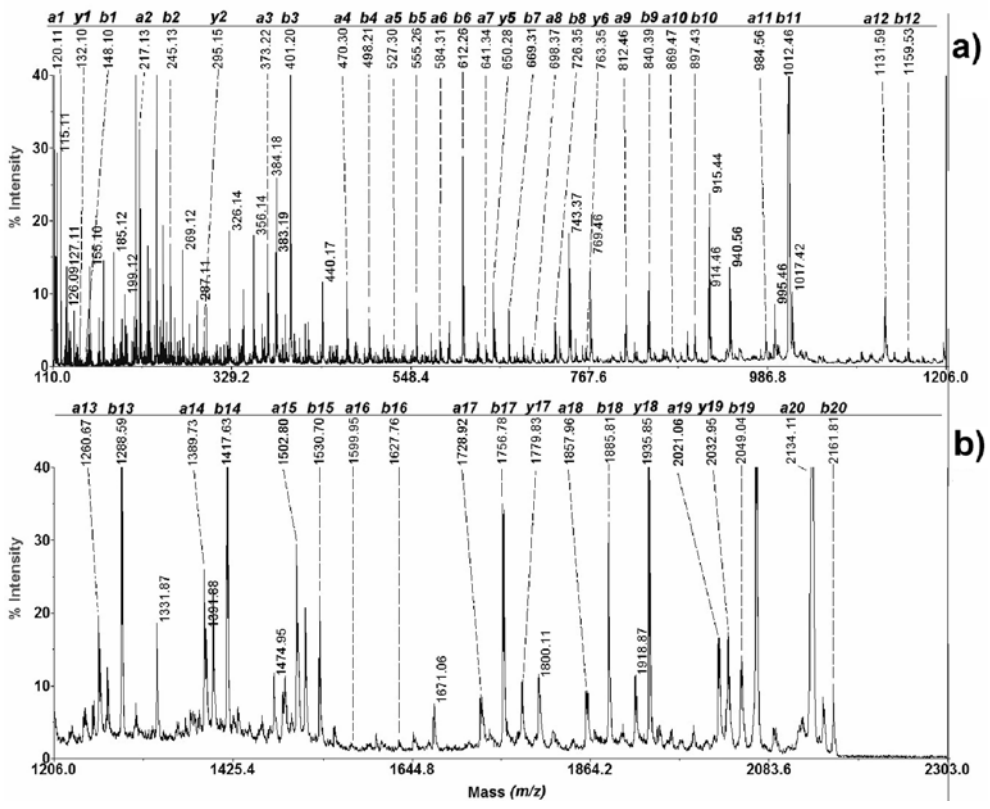

Figure 1. MALDI-TOF/TOF tandem mass spectrum of bivalirudin precursor ion (*m/z* 2180.0) with denoted a-, b- and y-series ions in the mass range *m/z* 110-2303 split in half (a and b). Signals with signal-to-noise ratio 5 or higher were taken into consideration.

formation of this type of ions, and y-series ions ladder sequence was incomplete on several places (y_3-y_4 and y_7-y_{16}) in spite of theoretical predictions. The rules set for unambiguous sequence determination can be more flexible in some cases, for example, b ions after high energy collisional activation can undergo further dissociation to form a ions, with immediate loss of carbon monoxide. Bivalirudin example of unambiguous peptide sequence determination is a clear cut illustration of precise, accurate and complete sequence analysis (*m/z* error with external calibration was ± 0.15). Theoretically calculated values for obtained a-, b- and y-series ions are presented on Figure 2. For isobaric amino acids Ile15 and Leu20, satellite ions (d-a and w-a) were not reported, but they were detected in a separate MS/MS 2 kV experiment at elevated collision energy and amount of collision gas (air) in the collision cell.

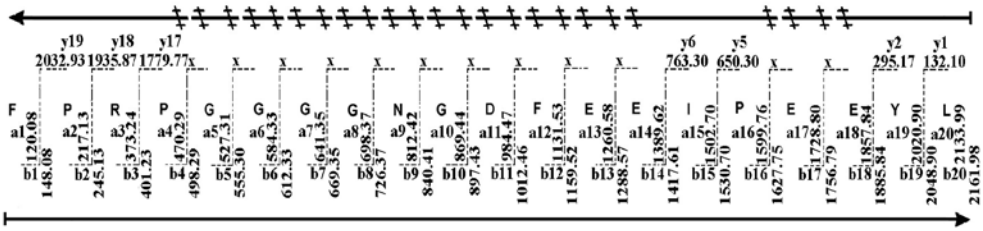

Figure 2. Calculated monoisotopic theoretical fragment masses for bivalirudin peptide fragmentation sequences**.** Masses for the fragments that were not observed in mass spectrum are not calculated (y_{3x}-y_{4x} and y_{7x}-y_{16x}). Signals in tandem mass spectrum for a- and b-series of ions were not interrupted by missing signals in opposite to y-series of ions (marked as x and ‡‡).

Unambiguous MS/MS analysis of intact exenatide

Exenatide is a 39 amino acid peptide with amidated C-terminus. Most of the laboratory enzymatic proteolyses are conducted in neutral or alkaline environment because physiological or slightly basic pH has optimal effect on enzyme activity providing high specificity and fragmentation yield. Sometimes, proteolytic enzyme is preoccupied in its activity by only one specific site in the peptide leaving the other sites of the same specificity uncleaved (miscleaved fragments). One of the problems arising during the time-consuming proteolysis procedure (especially observed for synthetic peptides) is structural decomposition of the specific groups, amino acids or fragments**.** Exenatide digested for 18 hours under basic conditions by trypsin was cleaved on T1, T2 (miscleved T1-2) and T3 covering 70% of the sequence. Fragment T4 was deamidated at C-terminus and therefore decomposed, and detected in trace amount in mass spectrum (denoted

with check mark on Figure 3). Deamidation can destabilize peptide structure and very often leads to the protein/peptide degradation (denoted with x mark on Figure 3). Changing the enzyme (Lys-C, Glu-C) or pH conditions did not improve sequence enzymatic cleavage coverage of Exenatide making unambiguous peptide analysis impossible in the first instance. Top-down analysis covered 32 out of 39 amino acid with a couple of gaps in two prelevant series of ions (b- and y-, Figure 4). Ions of y-series: y35, y34, y19, y17, y16, y14, y12 and y1 that represent amino acids T, F, L, I, E, L, G and S were not detected. Ions of b-series: b8, b25, b31, b33, b36, b37, b38 and b39 that represent amino acids S, W, P, S, P, P, P and S were not detected. However, overlapped b- and y-series ions sequences revealed the complete amino acid sequence justifying applied top-down approach instead of troublesome proteolysis.

CONCLUSIONS

The term "unambiguously peptide/protein determination" has been changed over the past 20 years [1,4]. Modern mass spectrometry has been approaching the unambiguous analysis of peptides, polypeptides and sometimes proteins under specific conditions (top-down approach). However, prosthetic groups and unfavorable charge distribution in peptides/proteins could aggravate the unambiguous analysis. Implementation of mobile and localized proton models

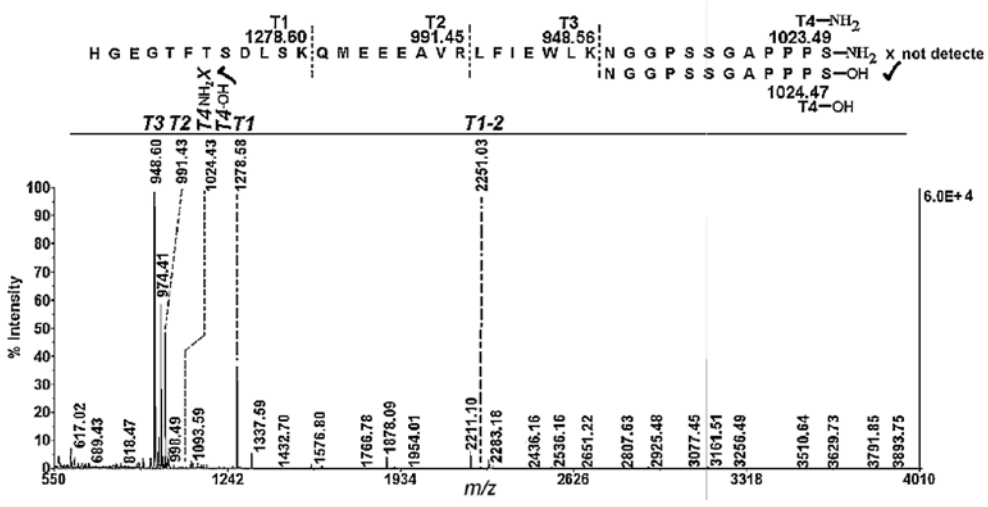

Figure 3. MALDI-TOF mass spectra of exenatide tryptic peptide fragments T1, T2, T3 and deamidated T4, as well as miscleaved T1-2 (-NH_2 denotes C-terminal amide).

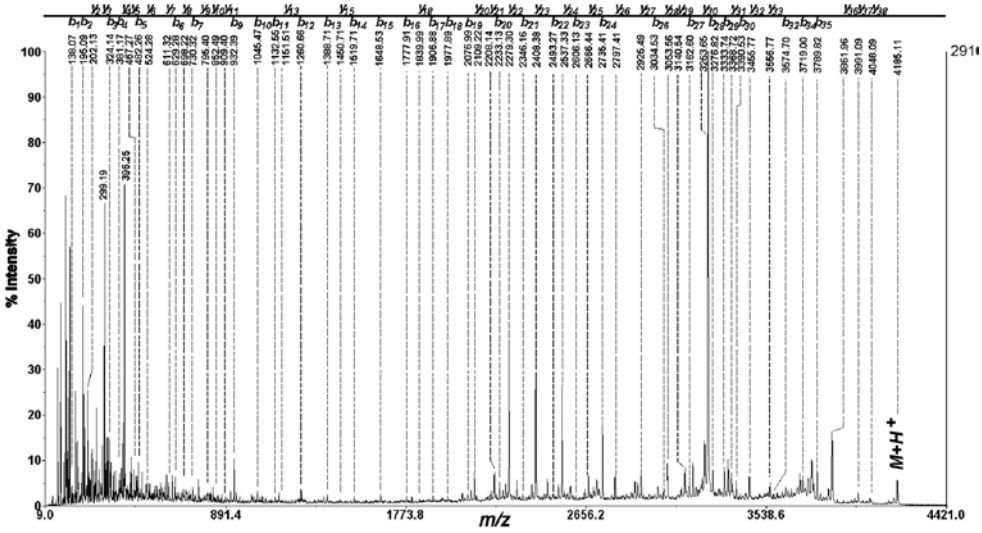

Figure 4. MALDI-TOF/TOF tandem mass spectrum of exenatide precursor ion (*m/z* 4185.11) with denoted b- and y-series ions in the mass range *m/z* 9-4421. Signals with signal-to-noise ratio 5 or higher were taken into consideration.

and selective derivatization could bridge the differences between complete and partial sequence analysis. Whenever it is possible, intact peptides should be analyzed without chemical addition of a group or groups that will enhance an overall mass spectrometry performance and irreversibly change the structure, except for tested and robust reactions where derivatization will not affect mass spectrum signals increment (e.g. *N*-terminal sulfonation mass spectrometry). An alternative choice, the derivatization approach that affects the ion masses sometimes is inevitable, especially in the cases where MS/MS spectrum of the intact peptide ion does not provide enough data for complete and unambiguous determination of amino acid sequence or prosthetic group(s) but modern mass spectrometry should be avoiding the derivatization concept.

Derivatization methods that do not change the masses of analyzed peptide product ions adjusted to the gas-phase MALDI fragmentation (SPITC) are more preferred than mass increment methods that change masses of precursor and product ions, e.g. Lys-tag (although not ideal, this approach is very efficient sometimes).

MALDI is a versatile and easy to use technique amenable to the implementation of theoretical knowledge in mass spectrometry fragmentation mechanisms into practice. Longer peptides (*m/z* > 5000) and proteins reluctant to fragmenta-

tion with a great number of modifications and prosthetic groups still remain a challenge for future technique and method development.

Acknowledgments

This manuscript was supported by the project of Ministry of Science Education and Sports 098-0982464-2393 and 098-0000000-3454.

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Sažetak

Određivanje aminokiselinske sekvencije farmaceutskih peptida MALDI-TOF tandemnom spektrometrijom masa

Iako su mehanizmi preddisocijacije, disocijacije i postdisocijacije iona izučavani dugi niz godina, postoji još cijeli niz mehanizama raspada iona koji se mogu korisno upotrijebiti u modernoj spektrometriji masa, proteomici i bioanalizama. Nedvojbena aminokiselinska analiza spektrometrijom masa počiva na ideji da samo jedan i kontinuirani slijed iona u tandemnom spektru masa može potpuno potvrditi cjelovitu aminokiselinsku sekvenciju i na taj način zadovoljiti zahtjeve nedvojbene analize peptida. Niz pravila kojima se definira cjelovita i nedvojbena analiza peptida tandemnom spektrometrijom masa u konačnici može unaprijediti iščitavanje rezultata analize i pouzdanost dobivenih podataka. Na teorijskim zasadama disocijacije iona u plinskoj fazi u ovom je radu prikazana cjelovita analiza peptida. Na modelnim farmaceutskim peptidima bivalirudinu i eksenatidu pokazano je kako se i bez raširene upotrebe derivatizacijskih tehnika (SPITC i Lys-tag) može ostvariti nedvojbena analiza, iako se modifikacije cisteina akrilacijom, metilacijom ili karboksimetilacijom pri tome ne mogu izbjeći.

Ključne riječi: MALDI-TOF/TOF; određivanje aminokiselinske sekvence; farmaceutski peptidi; bivalirudin; eksenatid