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Monitoring the neuropeptide metabolites by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Abstract

The developments of bio-analytical methods for analyzing bioactive peptides are of paramount importance. Neuropeptides and their bioactive fragments play a vital role in the regulation of many biological processes and diseases. This paper presents the use of matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) method for monitoring neuropeptides along with their degradation products in plasma samples from cancer patients. The neuropeptides focused in this study were β-endrophin, substance P, and bradykinin. The method involves the enzyme digestion of the neuroactive peptides followed by MALDI-MS sample preparation and subsequent acquisition of the MS spectral data. The mass spectral profile identifies most of the C-terminal and N-terminal peptides, and the mass accuracy was in the range of −1.68 to 1.46 Da with the mass spectrometer utilised. Analysis of the neuropeptide degradation patterns from the cancer patients were compared with the controls showed similar results. The study reveals that this approach can be used to identify the enzymatic digestion products of protein.

Keywords: Neuropeptides; Neuropeptide fragments; Metabolites; MALDI-TOF-MS; Cancer

1. Introduction

Many bioactive peptides are small peptides that exert potent biological actions in the respiratory, cardiovascular, endocrine, inflammatory, and nervous systems [1]. During the last decade, there has been increasing evidence which shows that numerous messenger molecules are involved in signaling in the nervous system, and most of these are neuropeptides. Several new neuropeptides have been discovered and recently Hökfelt et al. [2] published a detailed review on discovery of various neuropeptides. The functions of neuropeptides range from neurotransmitters to growth factors that have been implicated in the pathology of various neurological and psychiatric disorders. Most of these neuropeptides are initially synthesized as large, inactive precursors (propeptides). These are subsequently post-translationally modified to generate the final biologically active molecule and activated by limited proteolysis via a number of specific convertases. Neuropeptides are degraded by peptidases in the extracellular spaces. Complex neuropeptide signaling systems have been well studied and which showed to play an important role in many aspects of the cellular biology. Subsequently, these systems appear to have significant potential as targets for novel drugs. Several researchers reported that plasma peptidases are involved in the regulatory degradation of neuropeptides and cytokines [3,4].

Variations in the neuropeptide/and its fragment levels reflect the contribution in specific biological events and their direct functions. The interaction among different neuropeptide families might cause defects in the synthesis of bioactive molecules. Analysis of the various neuropeptide

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fragments from different families is essential for identifying the defects in neuropeptide synthesis, which may emerge as a result of the interactions of the peptides with the different neuropeptide families. Understanding the mechanism of neuropeptide degradation to their bioactive fragments is essential, which can be examined by the dynamics of neuropeptide signaling. This knowledge provides a better understanding of the metabolic pathways of neuropeptides that may lead to the design more effective drugs. Investigating the role of neuropeptides in disease states is complicated on account of the difficulties in measuring the levels of the bioactive fragments. The poor stability of neuropeptide due to degradation by a number of proteases is a major difficulty in quantification. Therefore, reproducible methodological approaches are needed for quantifying the levels of neuropeptides/and their fragments in various environments. In order to reveal the changes in peptide patterns and levels, several pioneering studies were focused to investigation and quantification of complete neuropeptide (entire sequence). However, there have been few studies on their fragments.

Previously, neuropeptides were studied by immunoassays protocols [5]. Other efforts in this direction include measuring mRNA expression levels and radioreceptorassays [6]. The limitations of these methods include the inability to unequivocally identify a specific neuropeptide, and a restriction in the number of peptides and neuropeptide fragments that can be analyzed simultaneously. Most of the above methods have relied on the specific antibody reactions. The main drawback of these immune-based methods is that a specific antibody is needed for each neuropeptide/and their fragments, which is a long and complicated process often involving measuring only one peptide at a time, after a lengthy and arduous purification procedure.

Mass spectrometry has emerged as a key enabling technology for the profiling of bio-molecules. One technology at the forefront of proteins and peptides field is matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) [7–11]. MALDI-MS, which is used in a variety of modes, provides information such as the molecular weight of a peptide, peptide mass mapping from a tryptic digest, and peptide sequencing. MALDI-MS is a suitable technique for the analysis of peptide mixtures resulting from enzymatic protein digestion. In addition, MS offers a novel rapid and sensitive methodology for the detection and quantification of multiple neuropeptides within a single complex sample specifically by its molecular mass.

Previously, we reported the capillary electrophoresis (CE) separation and analysis of neuropeptides in plasma using a multi-dimensional approach [12,13]. In the present work, an attempt was made to monitor the neuropeptide bioactive fragments in a complex biological matrix from the plasma samples of cancer patients. Bradykinin (BK), Substance P (SP), and β-Endrophin (BE) were the targeted neuropeptides examined in this study (Table 1). The methodology includes incubation of a neuropeptide in plasma samples, digestion by enzymes, a simple sample preparation step and MS peptide profiling of the proteolysis products of the neuropeptides. The aim of this study was to use a bio-analytical system that has a high level of sensitivity and specificity to detect and identify low therapeutic levels of neuropeptide fragments.

Table 1
Model neuropeptides studied

Neuropeptide	Sequence	Molecular weight (average mass)
Bradykinin	Arg^{1}-Pro^{2}-Pro^{3}-Gly^{4}-Phe^{5}-Ser^{6}-Pro^{7}-Phe^{8}-Arg^{9}	1060.22
Substance P	Arg^{1}-Pro^{2}-Lys^{3}-Pro^{4}-Gln^{5}-Gln^{6}-Phe^{7}-Phe^{8}-Gly^{9}-Leu^{10}-Met^{11}	1348.62
β-Endrophin	Tyr^{1}-Gly^{2}-Gly^{3}-Phe^{4}-Met^{5}-Thr^{6}-Ser^{7}-Glu^{8}-Lys^{9}-Ser^{10}-Gln^{11}-Thr^{12}-Pro^{13}-Leu^{14}-Val^{15}-Thr^{16}-Leu^{17}-Phe^{18}-Lys^{19}-Asn^{20}-Ala^{21}-IIe^{22}-IIe^{23}-Lys^{24}-Asn^{25}-Als^{26}-Tyr^{27}-Lys^{28}-Lys^{29}-Gly^{30}-Glu^{31}	3465.00

2. Materials and methods

2.1. Materials

The β-endrophin, substance P and α-cyano-4-hydroxycinnamic acid (a-HCCA) were purchased from Sigma (Sigma, St. Louis MO, USA). The bradykinin was obtained from Nova Biochem (San Diego, CA, USA). The sequencing grade trypsin, carboxypeptidases Y (CPY) and aminopeptidases M (AMP) was acquired from Boehringer Mannheim Gmbh Mannheim, Germany. All other chemicals and organic solvents were of analytical grade.

2.2. Experiments details

2.2.1. Carboxypeptidases Y reactions

To 1 μg ml^{-1} of the neuropeptide standards (dissolved in 50 mM citrate buffer pH 6.0) carboxypeptidases Y was added, and the reaction mixture was incubated for 30 min at 37 °C. During digestion, the enzyme:neuropeptide ratio was 1:100 (mol/mol). The reactions were quenched by adding trifluoroacetic acid (TFA) to 0.1%. A 2 μl aliquots of the digested mixture was mixed with the MALDI matrix (α-cyano-4-hydroxycinnamic acid), and then subjected to mass spectrometry.

2.2.2. Aminopeptidases M reactions

To 1 μg ml^{-1} of the neuropeptide standards (dissolved in 50mM Tris–HCl pH 7.5) aminopeptidases M was added, and the reaction mixture was incubated for 30 min at 37 °C. During digestion, the enzyme:neuropeptide ratio was 1:100 (mol/mol). The reactions were quenched by adding TFA to 0.1%. A 2 μl aliquots of the digested mixture was

mixed with the MALDI matrix (α-cyano-4-hydroxycinnamic acid), and subjected to mass spectrometry.

2.2.3. Trypsin reactions

Trypsin (in 0.1% TFA) digestion was carried out at 37 °C for 30 min by mixing the standard BE solutions, which has been prepared in 0.1M NH_4HCO_3 pH 8.0. During trypsinolysis, the enzyme:neuropeptide ratio was 1:50 (mol/mol). The reactions were quenched by adding TFA to 0.1% and subjected to mass spectrometry.

2.2.4. Human plasma neuropeptide degradation

Blood withdrawal and plasma preparations were performed as described elsewhere [13]. Synthetic neuropeptides were incubated with the dilute plasma (1:10) at 37 °C. After 30 min, the reaction mixture was mixed with the MALDI matrix and subjected to MALDI-MS target for mass spectral analysis.

2.3. MALDI-TOF-MS

The MALDI-TOF mass spectrometry measurements were performed using a HP G2025A system (Hewlett-Packard, Palo Alto, CA, USA) linear type time-of-flight mass spectrometer. The samples were prepared by mixing a 2 μL aliquot with 2 μL of the matrix solution (a saturated solution of a-HCCA in a 50% water/acetonitrile mixture with 0.3% TFA). One microliter of the sample mixture was spotted into a well of the sample plate and dried by a dried droplet or vacuum prior to mass spectrometry. The data for the 2 ns pulses of the 337 nm nitrogen laser were averaged for each spectrum in a linear mode, and positive ion TOF detection was performed using an accelerating voltage of 25 kV. The pressure in the ion chamber was maintained at between 1×10^{-7} and 4×10^{-7}Torr (1 Torr = 133.322 Pa). The ions generated by the laser pulses were accelerated typically to 28 KeV (1 eV = 1.602×10^{-19} J) energy and were ejected into the field free region of the TOF mass spectrometer. A dual microchannel plate was used to detect the ions. For data acquisition, the detector signal was pre-amplified and digitized by a digitizing oscilloscope (1 G samples/s) at a 500 MHz bandwidth (9350AM, LeCory, Chestnut, Ridge, NY, USA). The mass data was transferred to a PC for processing, which was accomplished using a data acquisition package (HP G2025A, TOF ware A 03.00 version 2.1, Pittsburgh, PA, USA). The MS system was externally calibrated with a peptide mixture containing Angiotensin II (monisotopic mass: 1046.5 Da, average mass: 1047.2) and ACTH 18–39 fragment (monisotopic mass: 2465.1 Da, average mass: 2466.7). Mass spectra were obtained by averaging 30 laser shots and repetitions of MALDI measurements were made for each sample to evaluate the reproducibility. All spectra were acquired with exactly the same instrument parameters (e.g., laser influence, number of laser shots, acceleration voltage).

3. Results and discussion

The peptidases have been involved in the biosynthesis of an active peptide. The enzymatic processing and degradation of several neuroactive peptides have been shown to undergo enzymatic conversion to their neuroactive fragments. Neuropeptides and peptidomimetics have a great potential to be used to treat many disease states such as cancer, HIV/AIDS, Alzheimer's disease and pain with biological actions rely on complete peptide sequences, including their fragments in some cases. Therefore, an analysis of these peptides is an important issue to address their effects in the disease state. Many methods are available for detecting, monitoring and quantifying the bioactive peptides in various tissues of the body. The main obstacle in neuropeptide analysis is peptidase degradation in complex bio-fluids. In order to determine the utility of the mass spectrometry technique on bio-molecules, a strategy for detection and identification of neuroactive peptide fragments was employed using MALDI-TOF-MS. Three different enzymes, CPY (release a C-terminal amino acid), AMP (releases N-terminal amino acid) and trypsin (cleaves peptide bonds specifically on the carboxyl side of lysine and arginine amino acid residues) were chosen to examine their action on the degradation of the targeted neuropeptides. The molecular masses of the peptide fragments obtained when the neuropeptides were incubated with the respective enzymes at specific time were identified using their *m*/*z* values.

3.1. Bradykinin

Bradykinin is a linear peptide with a wide spectrum of biological actions, and has been implicated in a variety of physiological and pathophysiological processes. The cardiovascular and inflammatory effects of BK are mediated by the activation of the B2 receptors [14,15]. Bradykinin digestion in human blood plasma or serum results in several products [16,17], which have specific biological activities [18,19] and this nonapeptide degradation profile has been investigated by many researchers [20,21]. In this study, in order to detect the BK fragments, the BK proteolysis reactions were carried out as explained in the experimental section and the resulting reaction mixture were subjected to MALDI-MS. The proteolysis products were identified by the *m*/*z* values. Table 2 summarizes the BE peptide fragments identified. Fig. 1A and B shows the mass spectrum of the BK metabolism with CPY and AMP, respectively. In both cases (CPY and AMP), the mass spectrum demonstrates that six amino acids were released at the studied conditions. Fig. 1C shows the mass spectrum of the BK degradation pattern in human plasma samples. It can be seen that BK hydrolysis results in the identification of all most all N-terminal and C-terminal peptides. The incubation of BE in human plasma resulted in four C-terminal (1–9), (1–8), (1–7), (1–5) and one N-terminal (6–9) fragments. The fragments identified in the plasma samples were consistent with the reported data.

Table 2
MALDI-TOF-MS masses of the bradykinin fragments

Bradykinin sequence	From–to	Enzyme	$[M + Hl^+]$	
			Calculated	Found
RPPGFSPFR	1–9		1060.22	1060.21
RPPGFSPF	1–8		904.05	904.21
RPPGFSP	1–7		756.88	757.74
RPPGFS	1–6	CPY	659.76	–[a]
RPPGF	1–5		572.68	573.68
RPPG	1–4		425.51	–[a]
RPP	1–3		368.45	367.51
RPPGFSPFR	1–9		1060.22	1061.61
PPGFSPFR	2–9		904.03	905.71
PGFSPFR	3–9		806.62	805.62
GFSPFR	4–9	AMP	709.80	709.15
FSPFR	5–9		652.75	654.21
SPFR	6–9		505.58	506.01
PFR	7–9		418.50	419.10

Mass: average mass.
[a] Not detected.

3.2. Substance P

Substance P is a member of the tachykinin family of neuropeptides [22]. It is a basic undecapeptide that is widely distributed in the peripheral and central nervous system. It has been reported that SP is involved in the pathophysiology of acute and chronic pain conditions [23]. Furthermore, there is also evidence that the SP fragments have biological activity. This tachykinin peptide is converted to a bioactive heptapeptide mimicking some of but opposing the other effects of the parent peptide. A number of peptidases, both cytosolic and membrane-bound, are known to cleave SP in several sites to form metabolites that exhibit a variety of biological and pharmacological actions. The SP fragments have been shown to exhibit both complementary and inhibitory actions relative to its parent peptide. In this study, in order to detect these fragments, the reaction mixtures obtained by SP incubation with CPY, AMP and blood plasma were subjected to MALDI-MS. The MALDI-MS products of the proteolysis were identified by the *m*/*z* values. The results of MS analysis of the CPY and AMP digestion products are shown in Table 3. The mass spectrum of the SP metabolism with CPY and AMP is shown in Fig. 2A and B, respectively. SP is degraded into six C-terminal (in case of CPY) and six N-terminal (in case of AMP) fragments. Fig. 2C shows the mass spectrum of the SP degradation pattern in the cancer patients plasma samples. From the results reported in Fig. 2C, SP was degraded into (1–10), (1–7), (1–6) and (1–4) fragments. The C-terminal fragment (1–4) was identified in the plasma sample was not detected in synthetic SP case [24]. This shows that the CPY activity was dominant in the human cancer plasma samples studied for the SP metabolism. Although similar SP fragments were found in the CPY digestion and plasma samples, the amount of the degradation products were different as indicated by the signal intensity.

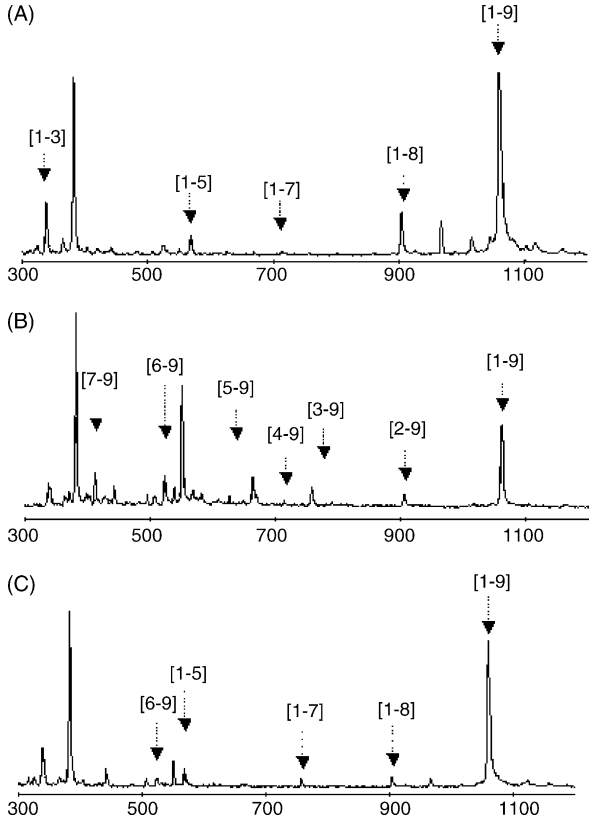

Fig. 1. MALDI-TOF-mass spectra of degradation of bradykinin: (A) carboxypeptidases Y; (B) aminopeptidases M; (C) cancer patient plasma sample.

3.3. β-Endrophin

Human β-endorphin is a 31 residue long peptide with a molecular mass of 3465 Da (average mass). Knowledge on the proteolysis of endorphin and the other peptides is

Table 3
MALDI-TOF-MS masses of the substance P fragments

Substance P sequence	From–to	Enzyme	$[M + Hl^+]$	
			Calculated	Found
RPKPQQFFGLM	1–11		1348.62	1349.00
RPKPQQFFGL	1–10		1217.45	1218.11
RPKPQQFFG	1–9		1104.29	1105.16
RPKPQQFF	1–8	CPY	1047.24	1047.92
RPKPQQF	1–7		900.06	900.80
RPKPQQ	1–6		752.89	753.87
RPKPQ	1–5		624.76	625.17
RPKPQQFFGLM	1–11		1348.62	1348.32
PKPQQFFGLM	2–11		1173.44	1172.41
KPQQFFGLM	3–11		1076.32	1076.61
PQQFFGLM	4–11	AMP	948.15	–[a]
QQFFGLM	5–11		851.03	850.00
QFFGLM	6–11		722.03	723.14
FFGLM	7–11		594.77	594.97
FGLM	8–11		447.60	447.32

Mass: average mass.
[a] Not detected.

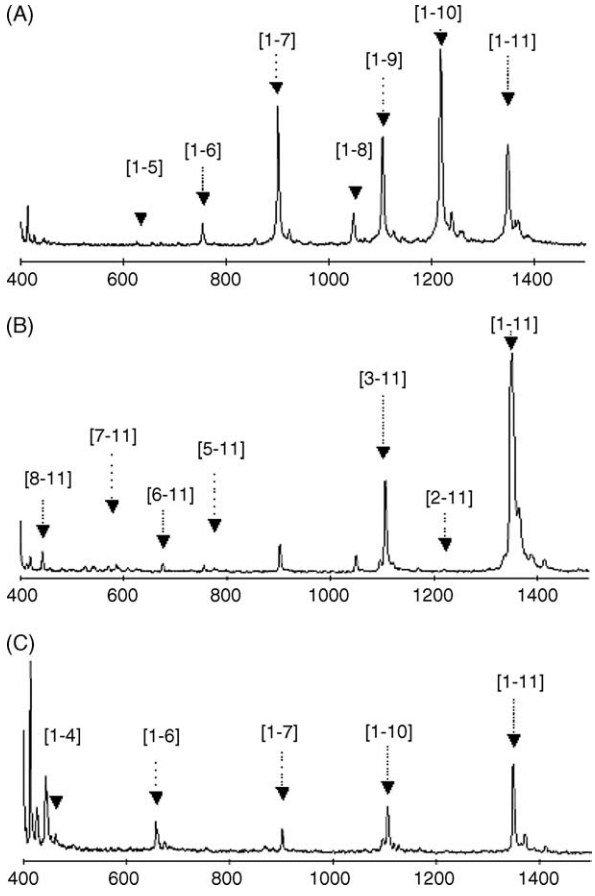

Fig. 2. MALDI-TOF-mass spectra of degradation of substance P: (A) carboxypeptidases Y; (B) aminopeptidases M; (C) cancer patient plasma sample.

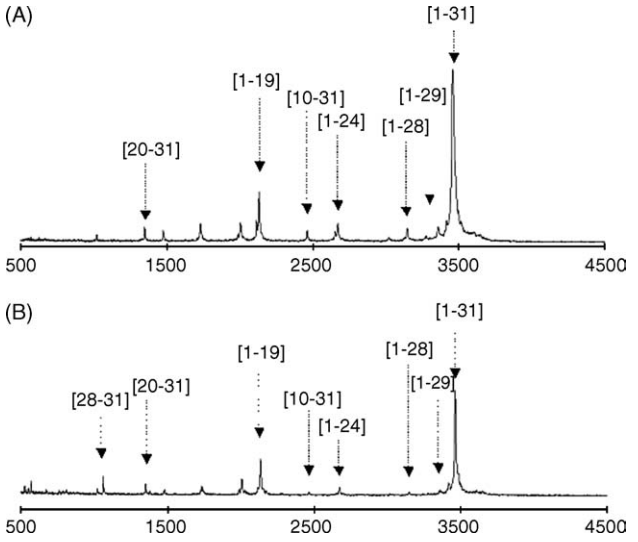

Fig. 3. MALDI-TOF-mass spectra of degradation of β-endorphin: (A) tryspin; (B) cancer patient plasma sample.

vital for clarifying the possible endogenous metabolic patterns and for optimizing the assays applied to neuropeptide analysis. Elevated BE plasma levels have been reported in certain endocrine tumors and in pregnancy [25,26]. Besides BE, their fragments also have biological activity. Along with the fragments (1–19) and (20–31), it retains biological activity in the guinea-pig myenteric plexus and inhibits morphine, respectively [27,28]. In this study, the degradation of synthetic human β-endorphin by a trypsin was examined using MALDI-TOF-mass spectrometry. From these mass spectra several major BE metabolites were identified based on their molecular ions. The mass spectrum of BE trypsinolysis and the BE degradation pattern in the cancer patients plasma samples are shown in Fig. 3A and B, respectively. Table 4 shows the MALDI-MS molecular mass of the BE fragments identified. MALDI-MS analysis yielded six significant peaks representing the loss of amino acids from the C- and N-terminals. Following tryptic digestion, MS identifies the masses of six BE truncated peptide fragments. The fragments formed were (1–29), (1–28), (1–24), (10–31), (1–19) and a C-terminal fragment (20–31). Incubation of BE with the plasma samples cleaved BE into several BE fragments as in the mock. Interestingly, a weak BE fragment (28–31) signal was observed in the plasma, which was not observed in the mass spectrum of the synthetic BE [29]. The signal intensity observed in the plasma samples were weak compared with the mock control, which may likely be due to the weak ionization efficiency.

MALDI-TOF-MS and neruopeptidomics is a platform that is gaining widespread use in drug development research. The importance of neuropeptides and their fragments is consequence of the key role that the peptides play in establishing the biological phenotype in both the healthy and diseased

Table 4
MALDI-TOF-MS masses of the β-endorphin fragments

β-Endorphin sequence	From–to	Enzyme	$[M+HI^+]$	
			Calculated	Found
YGGFMTSEKSETPLVTLFKNAIVKNAHKKGQ	1–31	Trypsin	3465.00	3465.91
YGGFMTSEKSETPLVTLFKNAIVKNAHKK	1–29		3276.12	3277.01
YGGFMTSEKSETPLVTLFKNAIVKNAHK	1–28		3148.16	3148.57
YGGFMTSEKSETPLVTLFKNAIVK	1–24		2672.48	2672.58
SETPLVTLFKNAIVKNAHKKGQ	10–31		2463.97	2462.51
YGGFMTSEKSETPLVTLFK	1–19		2133.08	2133.78
AIVKNAHKKGQ	20–31		1348.56	1348.81
KKGQ	28–31		623.71	623.81

Mass: average mass.

state. The repertoire of the plasma peptides changes with the disease state. This is specifically related to cancer and is an important issue when addressing cancer-related questions. Initial studies on the neuropeptide profiles have been defined in some diseases states, but many of these studies were limited to their neuroactive fragments.

Profiling the neuropeptides and their fragments will be useful for identifying the various cancer stages, which is a promising way to investigate the disease-related changes and research in this area is continually expanding. The method used in this study is sufficiently robust and reliable to allow specific questions regarding neuropeptide profiling in cancer or individual diseases to be addressed. Mass spectrometric analysis of neuropeptide and their fragment profiling information can be used in peptide-based therapy for the cancer development and progression stages. The main advantage of MALDI-TOF-MS is that it overcomes the need for the immunoassay approach for detecting and analyzing of neuropeptides in biological samples such as plasma, and brain tissue samples. In the immunoassay approach, antibodies are used to bind to the neuropeptides and subsequent analysis of neuropeptides, which are produced and raised against the whole sequence. However, there are no antibodies for the neuropeptides fragments to perform the immune-based reactions. Therefore, mass spectrometry has the main advantage of being able to detect and analyze the neuropeptides fragments without the use of antibodies. In contract to other neuropeptide analysis techniques, there is no need for precautions against sample degradation during preparation.

4. Conclusion

In this study, the MALDI-TOF-MS methodology was adapted for the direct monitoring of neuropeptides in the cancer patients plasma samples. The analytical strategy detailed above demonstrates the utility of MALDI-TOF-MS for monitoring the bradykinin, substance P, and β-endrophin fragments. These results suggest that a combination of enzyme digestion and MALDI-TOF-MS is an effective and convenient method for analyzing of the C- and N-terminal sequence of neuropeptides. The results reported herein show that MALDI-TOF-MS, in comparison with other methods, has advantages for the determination or confirmation of neuropeptide fragments, and the approach described is an excellent tool for monitoring the neuropeptides fragments within the biological samples. Gathering the profile of the molecular weights of the various neuropeptides between the normal and disease state present in a sample will provide a wealth of information for the discovery of novel neuropeptides and information on the degradation pattern of bio-active peptides can contribute to the formation of a new drug and biomedical applications. Future work will be focused on quantifying these neuropeptide fragments based on previously described MALDI-TOF-MS quantification procedure [30].

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