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Review

Quantitative analysis of methionine enkephalin and β-endorphin in the pituitary by liquid secondary ion mass spectrometry and tandem mass spectrometry

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Abstract

This manuscript reviews the use of an off-line combination of liquid chromatography (LC) and mass spectrometry (MS) to quantify endogenous neuropeptides in biological tissues and fluids, and tandem MS (MS/MS) to optimize the molecular specificity of the quantification of native peptides. Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to purify selected endogenous neuropeptides from biological tissues and fluids. Liquid secondary ion MS (LSI-MS), also known as fast atom bombardment (FAB), is used to desorb and to ionize the peptide. The corresponding stable isotope-incorporated synthetic peptide of each peptide is used as the internal standard (I.S.) for quantification. The measurement of methionine enkephalin (ME) and of β-endorphin$_{1-31}$ (BE) in the human pituitary is described. This analytical method offers the highest molecular specificity for the measurement of a fully post-translationally modified peptide. © 1998 Elsevier Science B.V.

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Contents

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1. Introduction

1.1. Objective

This review focuses on the use of an off-line combination of liquid chromatography (LC) and mass spectrometry (MS) to quantify endogenous neuropeptides in biological tissues and fluids. Gel permeation (GP), solid-phase extraction (SPE), and reversed-phase high-performance liquid chromatography (RP-HPLC) are used to extract and to purify selected endogenous neuropeptides from biological tissues and fluids. Liquid secondary ion-MS (LSI-MS), also known as fast atom bombardment (FAB), is used to desorb and to ionize the peptide. The corresponding stable isotope-incorporated synthetic peptide of each peptide is used as the internal standard (I.S.) for quantification; usually, four to five ^{2}H-atoms are incorporated into the I.S. peptides. Tandem MS (MS/MS) optimizes the molecular specificity of the quantification [1–5]. The measurement of methionine enkephalin (ME) and of β-endorphin$_{1-31}$ (BE) is described.

Many papers in the scientific literature discuss the chromatography of synthetic neuropeptides, and of synthetic neuropeptides that have been added to and extracted from a biological matrix and, in general, a post-HPLC detector, such as radioimmunoassay (RIA) and radioreceptorassay (RRA), is used.

The study of endogenous neuropeptides is especially important to clarify the understanding of physiological or pathological processes, such as pain perception (trigeminal tissues and CSF) or human pituitary tumor formation (pituitary tissues). Human tissues and fluids are the primary source of the neuropeptides that are discussed here, and animal tissues and fluids constitute a secondary source. Table 1 contains the list of tissues and fluids in which endogenous peptides have been measured in this laboratory with RIA, RRA, MS, and MS/MS.

This paper is not an exhaustive literature review, but rather focuses on one specific experimental approach to quantify two biologically important neuropeptides, ME and BE. The qualitative analysis (the determination of the amino acid sequence of the peptide) is performed before the quantitative analysis of ME or BE in biological tissues and fluids.

In general, ME and BE located in the brain, pituitary, and cerebrospinal fluid (CSF) are the targets for these studies. Those anatomical locations have been chosen because they contain these intact neuropeptides (and their corresponding OPCPs and large precursors), and do not usually involve any extensive metabolic degradation of the peptides, compared to the metabolism that would occur in blood, for example. These biologically active peptides derive metabolically from a large precursor protein molecule; for example, several new opioid peptide-containing proteins (OPCPs) have been detected [6].

Table 1
Biological tissues and fluids studied

A. Pituitary
B. Trigeminal system
● Ocular branch (cornea)
● Maxillary/mandibular branches (tooth pulp)
C. Amygdala/hippocampus (epilepsy)
D. Placenta
E. Cerebrospinal fluid
● Alzheimer patients
● Low back pain
● CSF peptidase activity
F. Saliva
G. Blood
H. Canine brain regions

1.2. General principles

It is important to put this specific analytical approach into the broader context of the study of peptides because the goal of a particular study plays a key role in the design of the experimental approach for peptide analysis. For example, four different goals exist for the qualitative analysis of an HPLC-purified peptide. (1) Elucidate the amino acid sequence, for the first time, of an unknown native peptide. (2) Confirm the amino acid sequence of a synthetic peptide. (3) Elucidate the amino acid sequence of a peptide that has been produced by the proteolysis of a synthetic peptide that has been added to a biological fluid or tissue. (4) Elucidate the amino acid sequence of a native peptide (such as ME, for example) each and every time that the peptide is extracted from a biological tissue and analyzed.

The goal for a particular study defines the level of chromatographic resolution needed vis-a-vis the molecular specificity of the post-HPLC detector. For example, when studying a synthetic peptide that has been added to a biological matrix, a molecular mass (M_r) determination by MS may suffice. However, when studying a native peptide, amino acid sequence data are needed. Also, sensitivity is generally not a problem when analyzing a synthetic peptide (goals 2 and 3).

Theoretically, if the molecular specificity of the chromatographic detector is 'low', then a high level of chromatographic resolution is required for the analysis of an endogenous peptide. Conversely, if the molecular specificity of the HPLC detector is high, then it is possible to decrease the required chromatographic resolution.

The purpose of this paper is to demonstrate the experimental approach that has been achieved to achieve the latter condition: namely, the use of off-line HPLC–MS/MS to optimize the molecular specificity of the quantification of native peptides [2,4,7].

1.3. Human pituitary

This research program has focused on opioid peptides in the pituitary. The opioid neuropeptidergic systems in the human pituitary are very important because of their multiple regulatory roles [8]. The hypothesis of one study is that a defective metabolism of those opioid systems may be a contributing factor to human pituitary tumor formation [4]. Thus, it has been necessary to process human pituitary post-mortem controls and to compare the peptide content with that amount in post-surgical tumor tissue. Several different human pituitary tumors have been studied, including non-secreting, prolactin-secreting, and others (ACTH-secreting, GH-secreting, etc.). The post-mortem controls are obtained as quickly as possible from the morgue, and the post-surgical tissue is obtained within minutes in the neurosurgical operating suite. Temperature is lowered as quickly as possible to minimize any metabolism, and the samples are stored at low temperature. Fig. 1 contains the analytical scheme that is used for the analysis of ME and BE in human prolactin-secreting pituitary tumors and controls [4], and represents the general features of the analytical procedure.

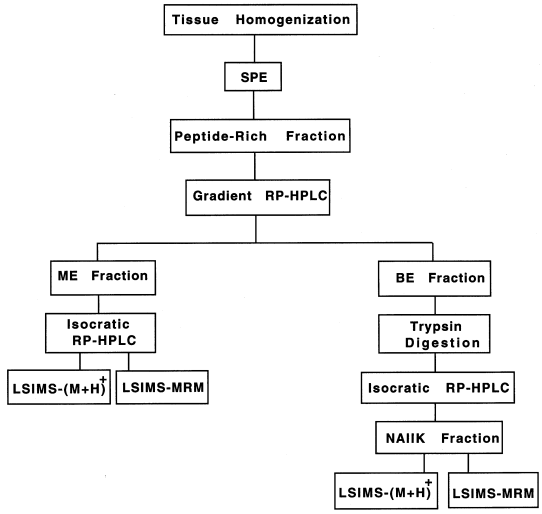

Fig. 1. Analytical scheme used for the qualitative and quantitative analysis of ME and BE in human pituitary tissue.

2. Solid-phase extraction (SPE) of peptides

As a first step in peptide extraction, the biological tissue (such as pituitary, tooth pulp, or canine brain tissue) is homogenized in acetic acid. For example,

previously frozen pituitary tissues were weighed quickly and homogenized individually in 10 ml 1.0 *M* acetic acid at 4°C [4]. The homogenate was incubated for 4 h at 4°C to ensure that the endogenous and exogenous peptides experience the same environment, and to precipitate large proteins and cellular debris. After centrifugation (31 000 *g*) for 30 min at 4°C, the supernatant was collected for SPE. A biological fluid such as cerebrospinal fluid (CSF) or serum can be directly applied to SPE [9–11].

The supernatant obtained after the centrifugation of the tissue homogenate contains several different biomolecules, such as proteins, peptides, carbohydrates, lipids, and others. SPE uses a pre-constructed mini-cartridge that is packed with a reversed-phase material, and is a fast and efficient method to isolate peptides from other molecules in that supernatant. The packing material inside the SPE cartridge is solid silica or a polymer, onto which is attached a long-chain hydrocarbon (usually C_{18}). Because peptides that contain ionizable groups are electrically charged in aqueous solution, they readily form an ion-pair with a buffer molecule. For example, in triethylamine–formic acid (TEAF) buffer, pH 3.0, the carboxylate group of a peptide may form an ion-pair with a protonated triethylamine molecule, and the protonated amino group of a peptide may form an ion-pair with a formate ion. The formation of ion-pairs increases the relative hydrophobicity of the ion-pair, which interacts with the hydrocarbon chains located within the pores of the SPE cartridge. Other relatively more hydrophilic molecules such as carbohydrates and salts are eluted by a low concentration of trifluoroacetic acid (TFA; 0.1%). The peptide ion-pairs that have been retained in the SPE cartridge are eluted with a buffer that contains a high concentration (50–100%) of an organic modifier, such as acetonitrile.

The loading capacity of the SPE cartridge is an important experimental aspect. The SPE cartridge used here (Sep-Pak Classic by Millipore-Waters, Bedford, MA, USA) contains 'picomole-equivalents of binding sites', and is suitable for the study of human pituitary tissues. The flow-rates of the loading and the eluting of the sample are both important to optimize for peptide analysis. Experiments with tritium-labeled ME and SP showed that optimum recovery (>92%) was obtained when the flow-rate was 75 μl/min [10].

3. High-performance liquid chromatography (HPLC)

3.1. Reversed-phase HPLC

Because nmol amounts of ME and BE are present in a human pituitary, off-line LC–MS was used in these studies. The volatile buffers that have been used for peptide HPLC include trifluoroacetic acid (TFA) and triethylamine–formate (TEAF) [12]. A volatile buffer is needed for RIA, RRA, MS, and MS/MS. The relative hydrophobicity of the peptide:buffer ion-pair plays a major role in the HPLC separation of the peptides. In some cases, smaller peptides that possess multiple charges and a high level of polarity are relatively difficult to separate by RP-HPLC because they elute at low concentrations of organic modifier. However, a volatile polyfluorinated acid such as heptafluorobutyric acid (HFBA) increases the relative hydrophobicity of the peptide:buffer ion pair, and thus also the retention time of the peptide. The HPLC analysis of tuftsin [13], a very polar tetrapeptide (TKPR), and a synthetic opioid tetrapeptide, Tyr-D-Arg-Phe-Lys-NH_2 (D-Arg^2-Lys^4-dermorphin amide = DALDA) [14], have both used an HFBA buffer.

3.2. Analytical HPLC columns

Two types of analytical HPLC columns have been used in this research program—octadecylsilyl (ODS) and synthetic hydrocarbon (polystyrene–divinylbenzene (PS–DVB) copolymer]) columns. Within certain pH limits (pH 2–8), ODS columns are chemically stable. However, bleed from an ODS column interferes with the FAB-MS analysis of a peptide in an ODS HPLC fraction [1] because FAB desorption is very sensitive to surface effects.

PS–DVB columns are chemically more stable, yield less bleed than ODS columns, and have been used in these studies after ODS. Gradient ODS chromatographic separations cover a wide range of hydrophobicity and M_r, and precede the isocratic

PS–DVB HPLC separation of a selected fraction from a gradient [1,4,7].

4. Analytical methods to analyze HPLC-purified peptides

Different methods have been developed to analyze HPLC-purified peptides; some are used to detect peptides and some to obtain the amino acid sequence of native neuropeptides. This section will discuss four methods that have been used in these studies (see Table 1).

4.1. Radioimmunoassay

RIA may be the most-used method to detect peptide-like immunoreactivity (peptide-li), and is based on the immunological reactions of targeted molecules and their antibodies. Along with immunohistochemistry, immunofluorescence, and enzyme-linked immunoassays (ELISAs), RIA has been used to provide a great amount of information on the anatomical localization of many different types of endogenous peptide immunoreactivity [15]. Theoretically, RIA displays a high level of specificity and detection sensitivity [16]. Most researchers agree with the aspect of detection sensitivity, because femtomoles of target peptide-li per analysis tube are generally detected.

However, the claim to molecular specificity remains open to question and experimental verification because RIA does not determine an amino acid sequence, but only detects an antigen–antibody (Ag–Ab) interaction and the radioactivity that is measured from the displaced antigen. Furthermore, in many cases, an antibody is raised to a synthetic peptide:protein immunogenic complex, because a peptide with a M_r of less than approximately 4000 does not possess sufficient immunogenicity [17]. For example, an antibody against ME was raised by chemically conjugating synthetic ME to a large carrier protein, such as thyroglobulin (TG), in a molar ratio ME:TG of ca. 30:1 to form an immunogenic complex [18]. The immunogenic complex is injected into an animal, where antibodies were raised to that complex, but not necessarily (and probably not) to ME.

Even though most RIA studies list the cross-reactivity of an antibody with a list of other peptides, that type of information cannot guarantee that, in an unfractionated biological sample, the antibody will react only with and completely with its putative target; nor, more importantly, does it ensure that, in an HPLC-purified fraction, the peptide with which it does interact possesses the appropriate amino acid sequence [19]. A specific amino acid sequence can never be guaranteed by an RIA measurement, and it is important to interpret all published RIA data with caution until the amino acid sequence of the target peptide is determined.

Nevertheless, even though RIA has limitations, it is commonly used as an HPLC detector. The effective use of the relatively limited specificity, but high detection sensitivity, of the RIA detection of an HPLC-purified peptide has been described [9,10,20,21].

4.2. Radioreceptorassay

RRA is another method that is used in opioid peptide analysis. RRA enables one to test the peptide:receptor binding activity to measure peptide-like receptoractivity (peptide-lr), and also to measure the number of receptors. RRA is based on the fact that there are several different types of opioid receptors, such as the μ-, κ-, σ-, δ-, and ϵ-receptors, which interact preferentially with morphine (μ), dynorphins (κ), enkephalins (σ, δ), and endorphins (ϵ), respectively [22]. However, no given peptide interacts with only one receptor, but rather with several different types of receptors, and with varying degrees of sensitivity and molecular specificity.

A synaptosomal-enriched fraction [23] from the canine limbic system has been used in the RRA of opioid peptides [24]. The limbic system contains the highest concentration of opioid receptors. Tritiated etorphin was used as the tracer ligand to compete with the binding of endogenous opioid peptides with the receptor preparation. In an RRA, the presence of an opioid peptide in an HPLC fraction is reflected as the receptor-binding activity. Usually, a sensitivity of

picomole amounts of opioid peptides is obtained with RRA.

Although RRA has a very low molecular specificity, it is nevertheless an effective method to comprehensively screen many HPLC fractions for the presence of different opioid peptides [25]. Such metabolic profiling [11,24–32] is very helpful because it can readily establish the relationships among peptide families, and it indicates those samples that must by funnelled to subsequent analysis. For example, after the presence of the target peptide is pinpointed by an RRA, that peptide can be further analyzed by another post-HPLC detector of higher molecular specificity, such as MS and MS/MS.

4.3. Mass spectrometry (MS)

4.3.1. General overview

MS provides the M_r of a peptide, which derives from the protonated molecule ion, $(M+H)^+$, of the peptide. LSI-MS (FAB) is used to desorb–ionize a peptide. Electrospray ionization-mass spectrometry (ESI) and matrix-assisted laser desorption–ionization (MALDI) are generally more useful for high molecular mass (M_r) compounds.

However, in a few cases, MALDI-TOF has also been used for the quantitative analysis of low-molecular-mass compounds and of bioanalytical compounds. Internal standards (I.S.) that were used included ^{13}C-labeled, ^{2}H-labeled, and peptide analogs [33]. In another study, three peptides were used and each peptide had a corresponding analog that was used for an internal standard [34].

To date, no ESI or MALDI quantitative data are available for peptides in the human pituitary.

MS/MS combines the analysis of the $(M+H)^+$ ion that is produced in one mass spectrometer (MS-1) with product-ion analysis in a second mass spectrometer (MS-2). The product-ion spectrum contains many, if not all, of the amino acid sequence-determining fragment ions of that peptide.

4.3.2. Instrumentation

All MS analyses discussed below were performed on an AutoSpec-Q tandem mass spectrometer from VG-Fisons Analytical (now MicroMass) (Altrincham, England). The tandem (MS-1, MS-2) geometry is: MS-1 = E_1BE_2; q (collision cell); MS-2 = Q, where E = electric sector, B = magnetic sector, Q = quadrupole. VG opus level 1.7 software was used to operate the MS, and to collect and analyze the data. The LSI-MS Cs^+ ion gun was operated at an energy of 35 keV. Glycerol was used as the matrix. The accelerating voltage was set at 8 kV. The mass resolution was ca. 1500 at m/z 393 (Cs_2I^+ ion). Each HPLC-purified peptide was reconstituted into 100 μl of 0.1% acetic acid in water–methanol (v/v = 1/1), and 10 μl was applied to the LSI-MS probe tip, which contained ca. 0.5 μl glycerol. The spectra were obtained at a scan-rate of 5 s/decade, and mass calibration was performed with the $Cs(CsI)_n^+$ ions ($n = 0–7$) to cover the range m/z 133–1952.

In the MS/MS Product-Q mode, LSI-MS was used for sample desorption–ionization. The precursor ion was selected by MS-1 with a resolution of ca. 1500, and MS-2 was scanned (5 s/scan over the mass range m/z 100–600) to obtain the product ion spectrum. The resolution of Q was adjusted to 1 at m/z 500.

4.3.3. Internal standards (I.S.)

The stable isotope-incorporated synthetic peptides, $(([^2H_4]Ile^{22})BE_{human,1-31}$ [3] and $(([^2H_5]Phe)ME$ [1], have been used for the LSI-MS MS/MS quantification of BE and ME, respectively, in human pituitary tissues (post-mortem and post-surgical tissues) [4]. Because a ^{2}H-incorporated peptide, compared to its corresponding endogenous peptide, has the same susceptibility to chemical and enzymatic degradation and to loss during tissue processing (mechanical) and peptide purification, and the same behavior during ionization and fragmentation, it is the best I.S. to use for the quantification of an endogenous peptide.

For the quantification of endogenous neuropeptides in human tissues (post-mortem and post-surgical tissues), 5 μg $(([^2H_4]Ile^{22})BE_{human,1-31}$ and 1 μg $(([^2H_5]Phe)ME$ were added to each tissue before homogenization [4,35]. The optimum ratio of I.S.: peptide was found to be between 1:4 and 4:1 [1]. The homogenate was incubated (4 h; 4°C) to ensure that the endogenous and exogenous peptides experience the same environment and for the precipitation of proteins and cellular debris. Once the I.S.:peptide ratio was determined by MS/MS (see below), the amount of the endogenous neuropeptide was calculated from a standard curve.

5. Selected example

This section will discuss the HPLC separation of endogenous ME and BE, and the qualitative and quantitative analysis of both peptides by MS/MS.

5.1. Gradient RP-HPLC of the peptide-rich SPE fraction

The peptide-rich fraction obtained from SPE was separated by a gradient RP-HPLC using a μBondapak ODS analytical column. Fig. 2 shows the gradient RP-HPLC chromatogram obtained from one human pituitary. The arrows indicate the retention times (obtained in a separate experiment to avoid any HPLC column contamination) of synthetic ME and BE (substance P (SP) will not be discussed here), and the corresponding horizontal brackets indicate the fractions that were collected for ME and BE. Synthetic peptides have a very reproducible retention time; the retention time of an endogenous peptide generally elutes a fraction of a minute earlier, possibly due to the effects that a relatively large amount of biological matrix material has on an HPLC analytical column. Because a second step of RP-HPLC is required and because the molecular specificity of the MS/MS method is so high, the 'collection window' is intentionally wide at this stage of separation to ensure the collection of all of the peptide.

5.2. MS/MS product-ion spectra of NAIIK (BE_{20-24}) and of ME

Before the quantification of BE (via its tryptic pentapeptide BE_{20-24}) and of ME, the amino acid sequence of each peptide was established in the product-ion spectra of ME and of BE_{20-24}. MS-1 selected the $(M+H)^+$ of the peptide, and MS-2 collected the product-ion spectrum, which contains the amino acid sequence-determining fragment ions that derived only from that $(M+H)^+$. This procedure is such an important step to remove ambiguity that we believe that it should be performed for each peptide and for every measurement; otherwise, the peptide name (=amino acid sequence) cannot be used.

The amino acid sequence-determining fragment ions (BE_{20-24}) are rationalized according to the peptide bond fragmentation scheme shown in Fig. 3A, in which the fragmentation of the BE_{20-24} (NAIIK) peptide chain is demonstrated. Fig. 3B contains the product-ion spectrum of synthetic peptide BE_{20-24}, and Fig. 3C shows the product-ion spectrum of the peptide that was extracted from a pituitary tumor. The amino acid sequence of the endogenous peptide extracted from human pituitary controls and tumors is thus established unequivocally as NAIIK by the presence of those amino acid sequence-determining fragment ions (compare the amino acid sequence-determining fragment ions and

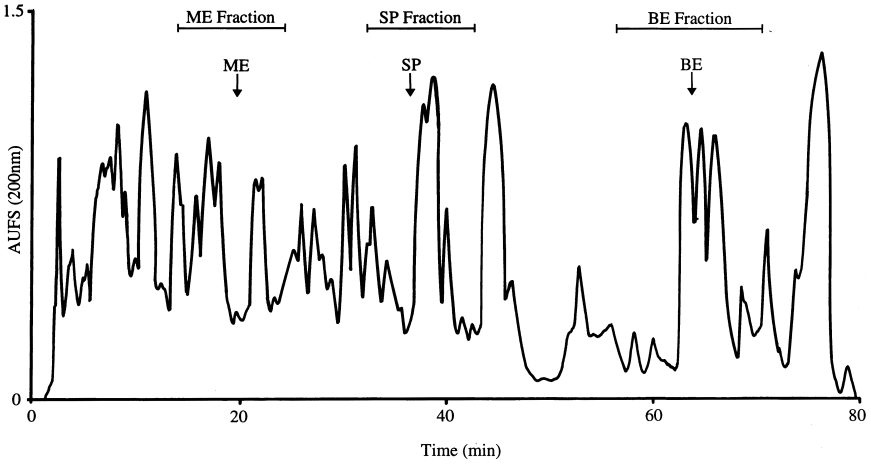

Fig. 2. Gradient RP-HPLC chromatogram of the peptide-enriched fraction obtained from a human pitutary sample. The arrows indicate the retention time of synthetic peptides, and the brackets indicate the collected peptide fractions.

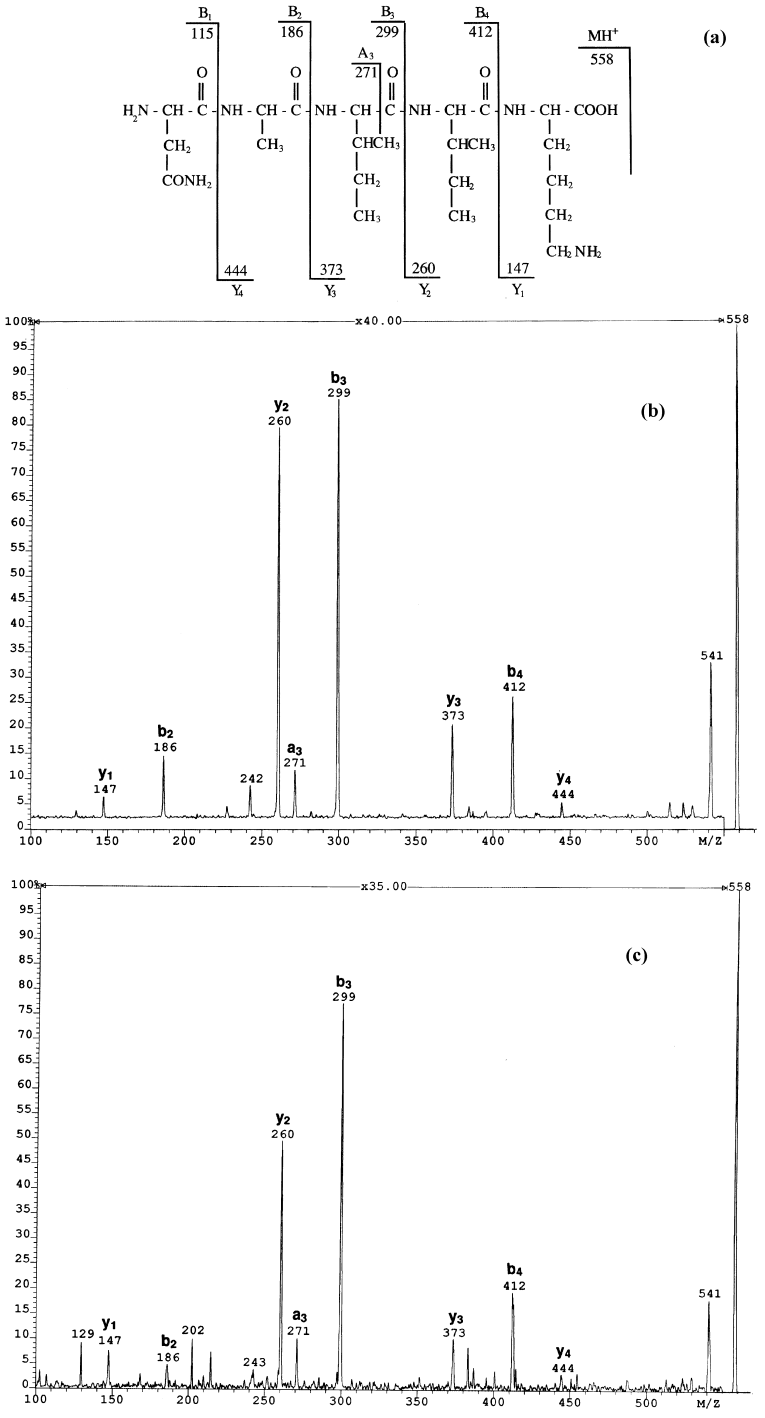

Fig. 3. (A) Fragmentation pattern of BE_{20-24}. The standard nomenclature of peptide bond fragmentation is used to designate the amino acid sequence-determining fragment ions. (B) Product-ion spectrum of synthetic BE_{20-24} (NAIIK). The MH^+ ion at m/z 558 of synthetic BE_{20-24} was selected as the precursor ion, and the product-ion spectrum was collected. (C) Product-ion spectrum of BE_{20-24} from a human pituitary tumor sample.

the equivalent product ion-spectra in Fig. 3B and 3C).

Even though the entire amino acid sequence of $BE_{human,1-31}$ was not obtained in this study, the molecular specificity of this method towards $BE_{human,1-31}$ was retained in the peptide extraction and purification procedures. For example, the fraction that eluted at the retention time of synthetic BE was collected from the gradient RP-HPLC, and the unique tryptic peptide NAIIK (BE_{20-24}) was chosen to represent BE. Therefore, the specific molecular linkage between NAIIK (BE_{20-24}) and BE was ensured throughout this study.

The amino acid sequence of endogenous ME was confirmed by the same procedures (data not shown).

5.3. Quantification of BE and ME by LSI-MS

In principle, the highest level of molecular specificity for the quantitative measurement of an endogenous peptide combines three experiments: (i) the measurement of the $(M+H)^+$ ion of the peptide; (ii) the measurement of either all of the N-terminal-containing or all of the C-terminal-containing amino acid sequence-determining fragment ions to ensure that the proper peptide is being quantified; and (iii) the use of a stable isotope-incorporated synthetic peptide I.S. The highlights of that process include the ionization of the peptide to form its corresponding protonated molecule, $(M+H)^+$ ion, the unimolecular or the collision-induced decomposition (CID) of that $(M+H)^+$ ion, and the corresponding amino acid sequence-determining fragment ions that are collected in the product-ion spectrum. Whereas the first and third items can be experimentally achieved relatively easily, all of the N-terminal and/or all of the C-terminal amino acid sequence-determining fragment ions may not be produced. The production of those ions depends on the desorption–ionization mode, the internal energy and fragmentation characteristics of the peptide under study, and the particular MS instrumentation that is used.

When using MS to quantify endogenous peptides, two important experimental parameters of the measurement are important: detection sensitivity and molecular specificity. Detection sensitivity can always be improved, and current MS methods allow one to measure compounds down to the zeptomole level. For example, oligopeptides that bind to the major histocompatibility complex (MHC) have been studied at high sensitivity [36].

On the other hand, molecular specificity is an even more important experimental parameter for the type of research described here, and for the neurosciences, chemical studies, and clinical studies. The reason for this importance is the fact that many conclusions are based upon only the immune- or receptor-based measurements of bioactive peptides. The amino acid sequence, which can only be implied in those other types of indirect measurements, must be clearly and experimentally determined each time that the peptide is being measured; however, those sequence data are generally not determined. For that reason, the 'structural resolution' that is provided readily by MS/MS has become an even more important goal than 'mass resolution'; that important aspect has been the major theme of this review [8].

To date, the highest level of molecular specificity for the quantification of an endogenous peptide has been achieved by MS/MS, which monitors the metastable transition that links the selected precursor ion with a unique product ion [17,25]. Generally, the $(M+H)^+$ of the peptide is chosen as the precursor ion, and one of its unique amino acid sequence-determining fragment ions is chosen as the product ion.

For BE quantification, channel 1 monitored the transition between the $(M+H)^+$ ion of BE_{20-24}(NAIIK) at m/z 558 and the N-terminal tripeptide fragment NAI– at m/z 299; and channel 2 monitored the transition between the $(M+H)^+$ ion of ($^2H_4$)NAIIK at m/z 562 and ($^2H_4$)NAI– at m/z 303. For ME quantification, channel 1 monitored the transition between the $(M+H)^+$ ion of YGGFM at m/z 574 and the N-terminal tetrapeptide fragment YGGF– at m/z 425; and channel 2 monitored the transition between the $(M+H)^+$ ion of ($^2H_5$)YGGFM and m/z 579 to ($^2H_5$)YGGF– at m/z 430. The ion current from the endogenous peptide was compared to the ion current from the I.S., and the ratio of those two ion currents was calculated. From the calibration curve, the endogenous peptide:I.S. ratio was obtained; that ratio was multiplied by the amount of I.S.; and the amount of endogenous peptide was calculated [35].

After the trypsinolysis of the BE fraction that was

collected in the gradient RP-HPLC, the mixture of tryptic peptides was separated with isocratic ODS RP-HPLC (Fig. 4). The arrow indicates the retention time of synthetic NAIIK, and the horizontal brackets indicate the fractions of NAIIK that were collected for BE quantification and for amino acid sequence determination of NAIIK. The inset shows the $(M+H)^+$ ions for endogenous NAIIK at m/z 558 and for the corresponding I.S. at m/z 562.

The ME fraction from gradient ODS RP-HPLC was separated on isocratic PS–PVD RP-HPLC. Fig. 5 shows the isocratic RP-HPLC chromatogram of endogenous ME. The arrow indicates the retention time of synthetic ME (obtained in a separate experiment), and the horizontal brackets indicate the fractions collected for ME quantification and amino acid sequence-determining ion analysis of ME. The inset shows the $(M+H)^+$ ions for endogenous ME at m/z 574 and for the corresponding I.S. at m/z 579.

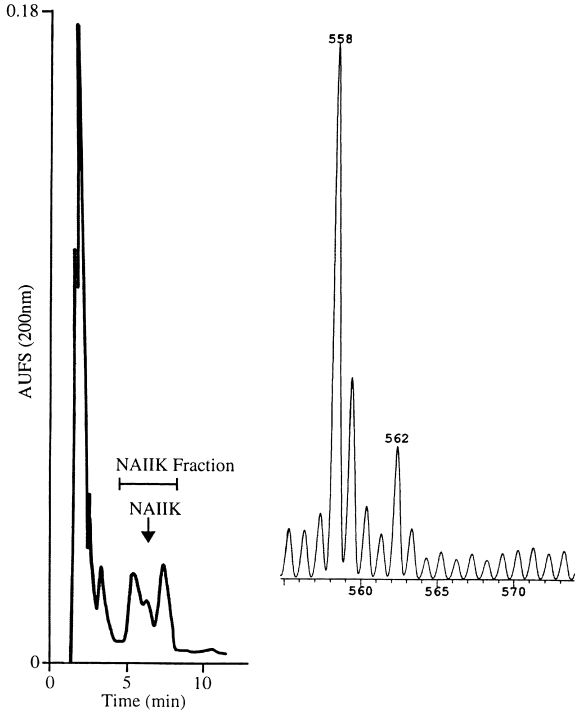

Fig. 4. Isocratic PS–DVB RP-HPLC chromatogram of the mixture of tryptic peptides that was produced by trypsin treatment of the BE fraction collected from the gradient ODS RP-HPLC separation. The mobile phase was 2.2% acetonitrile in 40 m*M* TEAF buffer (pH 3.0). The arrow indicates the retention time of synthetic NAIIK. The bracket indicates the NAIIK fraction that was collected for BE quantification and for the amino acid sequence-determining ion analysis of NAIIK. The inset shows the $(M+H)^+$ ion of NAIIK at m/z 558 and of the I.S. ($^2H_4$) NAIIK at m/z 562.

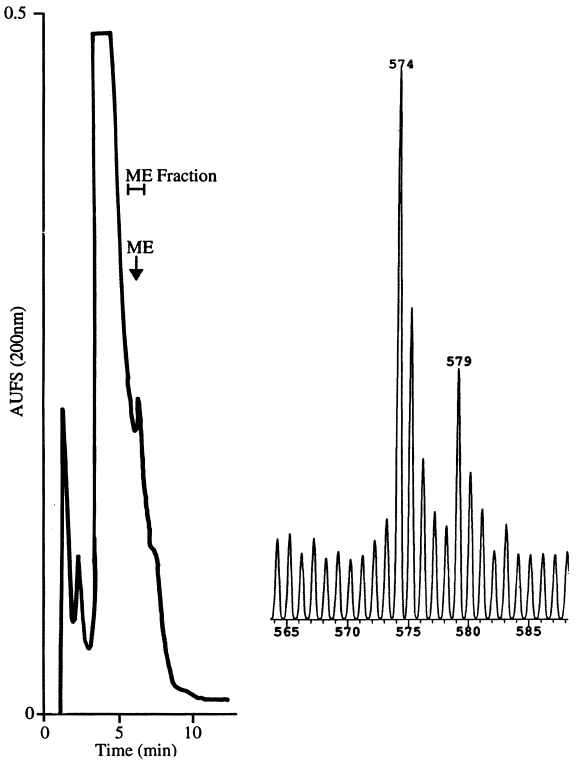

Fig. 5. Isocratic PS-DVB RP-HPLC chromatogram of endogenous ME. The mobile phase was 15% acetonitrile in 40 mM TEAF buffer (pH 3.0). The arrow indicates the retention time of synthetic ME. The bracket indicates the fraction that was collected for ME quantification and for the amino acid sequence-determining ion analysis of ME. The inset shows the $(M+H)^+$ ion of ME at m/z 574 and of the I.S. ($^2H_5$) ME at m/z 579

Table 2 contains the measurements of ME and BE in 10 post-mortem human pituitaries. The ME content was 76.6±6.2, and BE was 594±121 (S.E.M.) pmol/mg protein. The relatively high variation noted in those data derives not from the analytical method [1] but rather from the different endogenous peptide content in each individual.

The reproducibility of this analytical method is 2–5% [1]; the limit of detection of MS/MS measurements of synthetic enkephalins is fmol/amol [37].

Table 3 contains the measurements of ME and BE in prolactin-secreting tumors obtained from six

Table 2
Measurements of ME and BE in post-mortem human pituitary controls

Sample	BE (pmol/mg)	ME (pmol/mg)
1	—	56.4
2	—	67.3
3	—	53.9
4	—	87.7
5	332.6	100.5
6	671.1	92.5
7	815.6	67.8
8	1035.9	76.5
9	398.3	107.7
10	307.6	55.4
Mean	593.5	76.6
S.E.M.	121.1	6.2

—, not measured; PM, post-mortem.

females who had been treated with parlodel (promocryptine mesylate), which is often used before surgery. The ME was 1032±278, and BE was 450±253 (S.E.M.) pmol/mg protein. Those data demonstrate that the proenkephalin A system is regulated differently in those tumors versus controls (Table 2).

6. Conclusions

This manuscript has reviewed the quantitative measurement in human pituitaries of endogenous ME and BE by MS/MS. Two stages of HPLC (ODS, PS–DVB) were followed by LSI-MS–MS/MS. The amino acid sequences of native ME and BE were established by MS/MS. An H^2-incorporated synthetic peptide internal standard was used for quantification. This analytical method offers the highest molecular specificity for the measurement of a fully post-translationally modified peptide.

Table 3
Quantitative data of ME and BE in PRL-secreting human pituitary tumors

Female (on parlodel)	BE (pmol/mg)	ME (pmol/mg)
1	1526.8	1741.7
2	57.3	682.1
3	97.4	747.4
4	63.5	611.6
5	67.4	376.5
6	884.9	2035.5
Average	449.6±253.2	1032±278.1

Average, mean±S.E.M.
n.d., not detectable.

7. Abbreviations

Ag	antigen
Ab	antibody
B	magnetic sector
BE	β-endorphin$_{\text{human, 1–31}}$=YGGFMTSEK-SQTPLVTLFKNAIIKNAYKKGE
CID	collision-induced decomposition
CSF	cerebrospinal fluid
DALDA	D-Arg^2-Lys^4-dermorphin amide (Tyr-D-Arg-Phe-Lys-NH_2)
DTT	dithiothreitol
E	electric sector
ELISA	enzyme-linked immunoassays
ESIMS	electrospray ionization mass spectrometry
FAB	fast atom bombardment
GP	gel permeation
HFBA	heptafluorobutyric acid
I.S.	internal standard
LC	liquid chromatography
-li	-like immunoreactivity
-lr	-like receptorassay
LSIMS	liquid secondary ion-mass spectrometry
MALDI	matrix-assisted laser desorption–ionization
ME	methionine enkephalin (YGGFM)
MHC	major histocompatibility complex
MRM	multiple reaction monitoring
MS	mass spectrometry
MS-1	first mass spectrometer
MS-2	second mass spectrometer
MS/MS	tandem mass spectrometry
M_r	molecular mass
ODS	octadecylsilyl
OPCP	opioid peptide-containing protein
PS–DVB	polystyrene–divinylbenzene
q	collision cell
Q	quadrupole
RIA	radioimmunoassay
RP-HPLC	reversed-phase high-performance liquid chromatography
RRA	radioreceptorassay

SP	substance P (RPKPQQFFGLM-NH_2)
SPE	solid-phase extraction
TEAF	triethylamine–formic acid
TFA	trifluoroacetic acid
TG	thyroglobulin
TKPR	tuftsin (TKPR)

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