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REVIEW

PROFILING OF NEUROPEPTIDES USING GRADIENT REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH NOVEL DETECTION METHODOLOGIES

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1. INTRODUCTION

This chapter reviews chromatographic methods and detectors that can be used effectively to profile endogenous neuropeptides in biological tissues and fluids. The neuropeptides are separated by gradient reversed-phase high-performance liquid chromatography (RP-HPLC) and monitored with several detectors to profile the peptides; each detector focuses on a different molecular parameter. Peptide bond UV absorbance of the column eluate is monitored at 200 nm to detect peptides and proteins. Radioreceptor assay (RRA) is used to detect receptoractive (ra-) peptides that compete with selected radiolabeled ligands for binding to opioid receptors (mu, delta, sigma, kappa, etc.). RRA permits the screening of individual peptides in several opioid families because it is based on the affinity of receptors in a brain membrane preparation for specific ligands, where selection of the competing radioligands determines which peptides and peptide families will be detected. Immunoreactivity is the basis for a third detection system; radioimmunoassays (RIA) are used to detect individual peptides in fractions eluted from the RP-HPLC system. Two mass spectrometric (MS) techniques are also utilized; one to identify the protonated molecular ion, $(M + H)^+$, of a peptide and a second to quantitate the endogenous neuropeptides. This approach of detecting endogenous peptides with several detectors, with each detector focused on different molecular parameters of the peptides, provides the unique advantage of maximal molecular specificity. The screening aspect provides the diagnostically useful relationship among the peptides within and between opioid families.

Chromatograms of mixtures obtained from biological fluids and extracts were referred to as biological profiles [1]. Because the important requirement for the detection of compounds in biological mixtures is specificity, the detector used should measure compounds of interest and be insensitive to unrelated compounds.

Many peptide mixtures have been profiled, most commonly by HPLC with UV or electrochemical detection (ED). RIA has been used as a post-column detection method to further characterize the peptides [2]. Examples of peptides separated with these methods include peptic fragments of human immunoglobulin G [3], plasma angiotensins [4], peptide antibiotics [5], peptide hormones [6], neurophysins [7], and intestinal peptides [8].

Many investigators characterize the peptides they are measuring only in terms of the peptide's column chromatographic behavior and interaction with an opioid receptor preparation or an antibody. Indeed, many workers perform RIA without prior HPLC purification in the belief that the antibody used has total specificity. However, methods that combine HPLC, RIA, and RRA are necessarily limited in their specificity because of the following considerations. Peptide-rich fractions of biological tissue extracts contain a large number of different molecules, each with its own concentration. Even under optimal chromatographic conditions, it is extremely improbable that these molecules could be totally separated from all other compounds in that extract, each compound into its own discrete chromatographic peak, because the number of possible peptides that range in size from dipeptides to tridecapeptides is close to 7 billion (2! + 3! -- 13!). Even recognizing that a given tissue probably contains

only a very small number out of all of those theoretically possible peptides, a large fraction of peptides would still probably be present; therefore, the probability is low that HPLC separation would resolve all of the components in the tissue. Even if infinite HPLC resolution were achieved, the question of the molecular specificity of the detector would still remain.

The authors stress the fact that, even though all of the above-listed detectors, except for MS, are extremely useful and quite sensitive in screening for the presence of neuropeptides in the tissue and fluid extracts, they still lack the specificity needed to establish with confidence the molecular structure of each peptide present in the tissue. Only MS, particularly in the fast atom bombardment (FAB)-MS—MS, has the unique advantage of providing the highest level of molecular specificity, and its use leads to a high degree of confidence in the identity of the peptide measured.

1.1. Neuropeptides

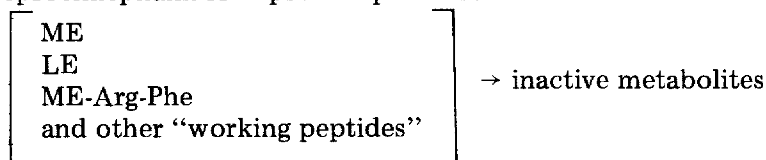
MS and accurate masses were used to elucidate the amino acid sequence of the first hypothalamic releasing factor, pyroglutamyl-histidyl-proline-amide (TRF) [9]. That study led to increased activity in brain neuropeptide research [10], and other releasing factors were elucidated.

The two pentapeptides, methionine enkephalin (ME = YGGFM) and leucine enkephalin (LE = YGGFL), were found to be endogenous ligands for the morphine (mu) receptor. Their structures were determined with the aid of MS [11]. These two pentapeptides derive from the proenkephalin A precursor protein molecule, which contains several copies of ME and one of LE [12]. While the enkephalins do bind to the mu receptor, it has been shown that they have a higher affinity for the brain delta receptors [13].

The proenkephalin B molecule is believed to be the precursor for the dynorphin ("dynamic morphine") family of peptides [14]. The dynorphins have extraordinarily high opioid activity and bind preferentially to the kappa receptor [15]. Beta-endorphin is a 31-amino acid (residues 61—91 of beta-lipotropin) opioid peptide [16] and is one of the neuropeptides derived from a third opioid family, the proopiomelanocortin (POMC) family [17]. The undecapeptide, substance P (SP) [18], a tachykinin with a putative role in nociception mechanisms [19,20], derives from the protachykinin precursor [21].

These precursor molecules in turn derive from larger gene products molecules via a metabolic process schematically illustrated below:

Preproenkephalin A → proenkephalin A →



These opioid peptidergic gene products are about 300 amino acids long, and may contain several copies of the individual target peptides [22]. The individual working peptides are derived from their corresponding precursor depend-

ing upon the metabolic demand that exists at a particular time in that organism. Much of the current work described in this review relates to the mobilization of specific peptide families in a tissue-specific manner (tissue-specific processing) and to the analytical methods used to monitor the change(s) in concentration of these peptides during or following nociception, stress, and tumor formation.

Peptides have several unique properties that combine to make that family of compounds a challenging object of study. Cells generally employ peptides to communicate effectively with other cells by means of transmitters, hormones, receptors, etc. [23]. The information content of a peptide is extraordinarily high as compared to other biological polymers such as a polynucleotide or polysaccharide and therefore, are best-suited to transmit specific information.

Even when compared to a computer word, the twenty naturally-occurring amino acids that combine to form peptides produce orders-of-magnitude higher levels of specific information content. Peptides are polymeric and consist of a linear sequence of monomeric units. For a polymer of n different monomers, $n!$ unique polymers with different monomeric sequences are possible. As one example, although there are $2^6 = 64$ possible six-bit computer words, there are $20^6 = 64\,000\,000$ possible hexapeptides. Thus, it is crucial to unambiguously determine the amino acid sequence of a peptide when measuring the endogenous content of that peptide in a biologic tissue extract [24]. Most analytical techniques such as RIA, RRA, or MS (in the limited protonated molecular ion mode) cannot convey the amino acid sequence of a peptide. Only when the amino acid sequence of the endogenous peptide is known, or whenever an amino acid sequence-determining fragment ion is used in the MS measurement procedure, can one be assured of the structure of the peptide and therefore place greater assurance in the analytical measurement of that peptide [24—26].

Several brain areas have been studied to determine their peptide content and the peptidergic pathways operating in the different regions of the brain. Thalamus, hypothalamus, caudate nucleus, pons medulla, hippocampus, midbrain, and amgydala are all screened by RRA for neuropeptide receptor activity [27] and/or by RIA for immunoreactive neuropeptides [28]. Pituitary tissue [29] and cerebrospinal fluid have been studied. Tooth pulp is also studied under normal and stressed conditions [30, 31]. Tumors of different origin are also screened in an effort to understand tumor genesis [29, 32].

2. NEUROPEPTIDE PROFILING

2.1. Tissues screened

When studying nociception or pain mechanisms, a convenient and appropriate tissue as a source for endogenous peptides is the tooth pulp; it is highly innervated and it is protected within a calcified tissue [33]. It is hypothesized that the only output from the nervous system in the tooth pulp is pain [34]. Therefore, we obtain tooth pulp from the canine model and from human patients who are being fitted for braces. We obtain human teeth normally (control), as well as after stress is applied via a transpalatal spring that exerts a known orthodontic force (g·min) on the teeth.

Cerebrospinal fluid (CSF) is another appropriate biologic fluid in which to measure endogenous peptides because the CSF is continuously produced by the choroid plexus, it circulates throughout the brain and spinal cord system, and it comes into intimate contact via intracerebroventricular circulation with internal brain regions such as the caudate nucleus [35]. Therefore, human CSF is obtained via lumbar puncture in a series of patients undergoing evaluation for lower back pain and facing potential surgery [36]. The focus of that study is to determine the pathophysiology of those patients whose pain is not diminished following pharmacological or surgical treatment.

Tumor tissue is appropriate for study of several peptide hormones because endocrine tumors generally produce high levels of hormones and peptides [29]. The same genetic apparatus and tissue-specific processing enzymes may be present in tumor tissue as they are in normal tissue, and analytical advantage can be taken of the increased amounts of individual peptides produced in tumors. Pituitary tumor tissue is obtained using the transsphenoidal neurosurgical procedure [29]. Human pheochromocytoma tissue has also been screened for proenkephalin A-derived peptide by HPLC coupled with ED [32].

It is imperative that the biological tissue and fluid be obtained as rapidly as possible to avoid production of artifacts via unintended metabolism of the peptide precursors and working peptides. One of the methods to effectively minimize production of artifacts is to rapidly lower the temperature of the tissue or fluid immediately following its acquisition by immersing the sample in liquid nitrogen and storing at -70°C until biological extractions can be performed. Microwave irradiation, enzyme inhibitors, and boiling have also been used to stop enzymatic degradation of neuropeptides. Micromolar concentrations of thiorpan, bestatin and captopril are used to inhibit enkephalinases, aminopeptidases and angiotensin converting enzyme, respectively [37].

Although the objective of these analytical measurements is to measure unambiguously the presence of peptides in individual subcellular compartments, that objective is relatively difficult to achieve; rather, it is experimentally more useful and expeditious to homogenize a neurosurgically-obtained tissue. Homogenization in an acid milieu will precipitate the larger proteins [38]. On one hand, if one is extracting the smaller peptides (LE, ME) for quantification purposes, this homogenization–precipitation procedure is acceptable. Further protein precipitation is achieved by adding acidified acetone [5 parts acetone–0.01 *M* hydrochloric acid (80:20) to 1 part homogenate]. On the other hand, for studies on the larger peptide/protein precursors, it is important to avoid the precipitation of proteins and precursors, and therefore homogenization at a neutral pH (water) is appropriate, with care being taken to avoid enzymatic degradation of the endogenous peptides.

At this early stage in the tissue processing, either internal standard for MS quantification purposes, or tritiated peptides for recovery studies are added. For MS, a stable isotope-incorporated peptide internal standard is added at this point; this internal standard may be a ^{2}H- or ^{18}O-containing peptide [39]. If the internal standard is an ^{18}O-containing peptide, then experimental care must be taken that the labile carboxyl exogenous ^{18}O does not back-exchange under acidic conditions with endogenous ^{16}O-species.

It is generally helpful to perform a prepurification step to remove non-

peptide compounds and to preferentially concentrate the peptides before HPLC separation. The supernatant remaining after protein precipitation is applied to a disposable octadecylsilica (ODS) minicolumn (Sep-Pak) that effectively and preferentially retains the endogenous peptides [38, 40]. Peptides are eluted from the column to produce a peptide-rich fraction. Trifluoroacetic acid (0.1%) is generally used as the aqueous buffer in the Sep-Pak procedure, with the organic modifier acetonitrile used to elute the peptides. If ^{18}O-containing peptides are present, a Tris–methanol system is used. A water–ethanol buffer system has also been used at near neutral pH [32]. This peptide-rich tissue extract is then applied to a C_{18} RP-HPLC column.

2.2. *RP-HPLC*

Neuropeptides are effectively separated by RP-HPLC. When profiling closely related peptides in the same size and/or hydrophobicity range, isocratic HPLC offers a simple and fast separation method. For example, proenkephalin A-derived peptides ranging from five to eight amino acid residues have been separated isocratically and detected by amperometric ED [32].

We have developed a gradient RP-HPLC scheme for the fast and facile separation of peptides possessing a wide range of hydrophobicity. Although peptides are generally quite polar due to the presence of zwitterionic termini, polar side chains and interchain hydrogen bonding, they elute quite well from a RP-HPLC column when an ion-pairing reagent is added to the aqueous buffer. The

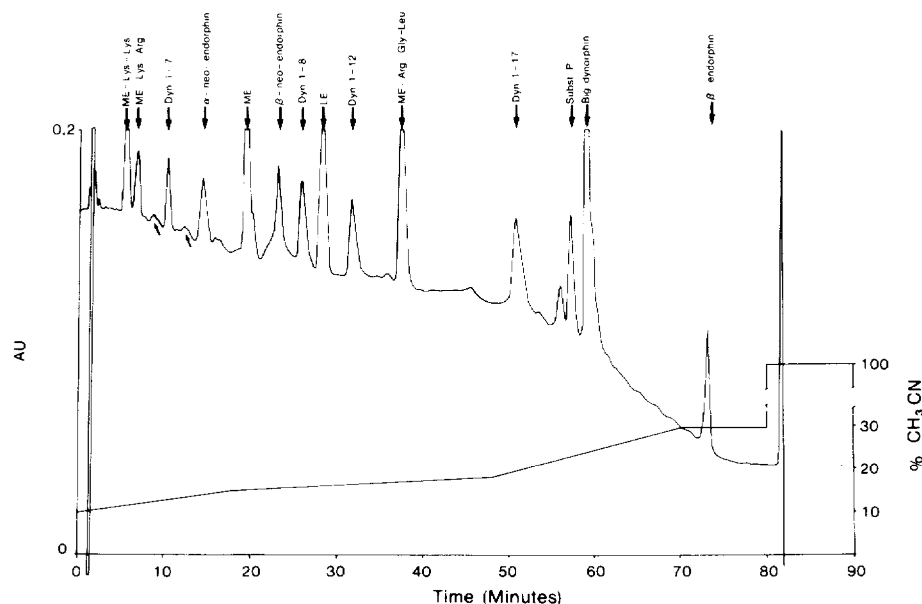


Fig. 1. Gradient RP-HPLC detection of synthetic peptides. Varian 5000 liquid chromatograph with 15-cm μBondapak column (Waters Assoc.). Flow-rate: 1.5 ml/min; 0.2 AUFS; UV monitoring: 200 nm; aqueous buffer: 0.04 *M* formic acid with triethylamine, pH 3.15 (TEAF); organic modifier: acetonitrile (CH_3CN); gradient profile is indicated as the solid line from 10 to 100% CH_3CN, indicated on the right hand axis. Arrows indicate some of the buffer peaks caused by the organic modifier step increases (see text).

peptide—buffer ion-pair possesses a reduced polarity, and more effectively interacts with the hydrophobic C_{18} side-chains in the analytical column [41]. Because we detect and quantify peptides of interest with several post-HPLC detectors, it is important that the HPLC buffer be volatile to minimize interference with subsequent peptide detection. We have developed a volatile triethylamine formate (TEAF) buffer consisting of 0.04 M formic acid adjusted to pH 3.15 with distilled triethylamine. Acetonitrile is used as organic modifier [42, 43].

The RP-HPLC gradient profile is illustrated in Fig. 1, which plots the percentage of acetonitrile on the right-hand axis. The chromatogram shows the elution of several synthetic peptide standards under these gradient conditions, monitored by UV at 200 nm. This chromatogram demonstrates the wide range of hydrophobicities of peptides that can be readily separated with our gradient system. The baseline shift observed as the percentage of organic modifier increases is due to the absorbance of the TEAF buffer at this wavelength.

Small peaks are observed regularly along the chromatogram as the gradient develops. These peaks occur in all blank runs. We rationalize that these peaks relate to the increases in the organic modifier concentrations as the gradient develops. Some of the formic acid and/or the triethylamine in the aqueous buffer may be retained within or on the surface of ODS-silica particles, and then elute as the percentage of organic modifier is increased. The organic modifier acetonitrile effectively disrupts intermolecular interactions. Due to the absorbance of the TEAF buffer at 200 nm, elution of the retained buffer increases the absorbance of the eluate and produces the periodic small peaks in the chromatogram, as illustrated by the arrows (Fig. 1).

The rationale of this phenomenon of regularly-spaced peaks can be proven experimentally by diluting (1:1) the organic modifier with the aqueous buffer and reprogramming the gradient, maintaining the gradient profile equivalent to the original one. In this case, the small peaks appear twice as often, reflecting the fact that there will now be twice as many step-increases in the organic modifier- buffer percentage to maintain the same rate of organic modifier increase in the gradient.

It is necessary daily to separate a mixture of standard peptides to calibrate the elution times of the peptides of interest. Although retention times show little variation, the standard chromatogram is useful in monitoring the condition of the column and other instrumental parameters, and it can also alert us to any problems with the instrument. However, extreme care must be taken to insure that no residual amount of the standard(s) is retained in the injector or in any part of the instrument hardware; retained standards could lead to artificially high results of endogenous peptides measured in a subsequent run. Our experience indicates that some sample could be retained in the injector and then be injected onto the column with the next sample injection. Fractions collected from blank runs immediately following these standard runs have shown receptor activity (ra-) and immunoreactivity (ir-). Because the standards are injected in microgram amounts and the level of neuropeptides in the tissue extracts is generally at the picomole (ng) level, a small amount of standard peptides retained in the injector and subsequently injected along with the sample in the next run could cause a major change in the levels of peptides quantified.

We assiduously avoid this retention problem by following each gradient run with an additional wash (10 min, 100% acetonitrile), during which time a volume of water greater than the volume of the injection loop (1.5 ml water for a 1-ml loop) is injected onto the column. After allowing sufficient time for the entire volume content of the loop to be injected into the column, a second injection with methanol is made in a similar fashion, and the column is washed with at least five column volumes of acetonitrile. Any retained sample is thus removed from the injector and eluted from the column with the organic modifier. In addition, the injector valve is kept in the inject position during the gradient run to permit the buffer to continuously flow through the injection loop. With all of these precautions, and after monitoring (RRA, RIA) blank runs following the elution of standards, we experimentally and routinely ensure that no ra- or ir-material elutes.

Figs. 2 and 3 show the RP-HPLC separation of the peptide-rich fractions derived from a human pituitary and a human tooth pulp, respectively. These samples are eluted under the same gradient RP-HPLC conditions as in Fig. 1, and these data illustrate the wide variation in the amount of UV-absorbing material present in these two tissues. The peaks observed in these chromatograms are predominantly peptidic in nature because they have been shown to be sensitive to proteolytic enzyme treatment [44].

It must be stressed again that endogenous neuropeptides are generally present at the low picomole level in the biological tissues studied and, therefore, we do not expect to observe discrete UV-absorbing chromatographic peaks. Furthermore, co-elution of an HPLC peak with a known peptide

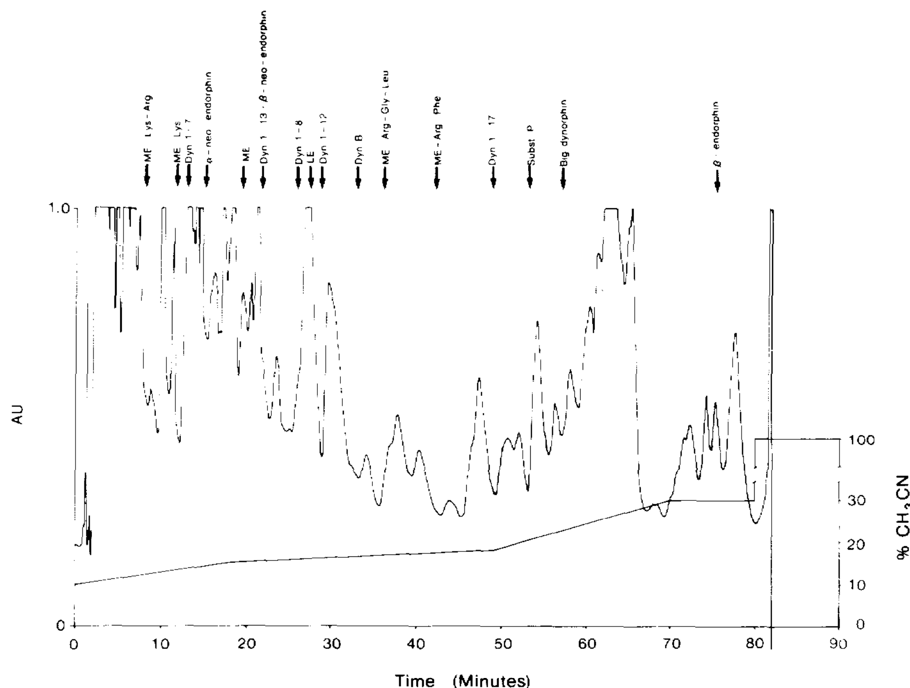


Fig. 2. Gradient RP-HPLC chromatogram of peptide-rich fraction from a normal pituitary tissue. 1.0 AUFS; other conditions as Fig. 1.

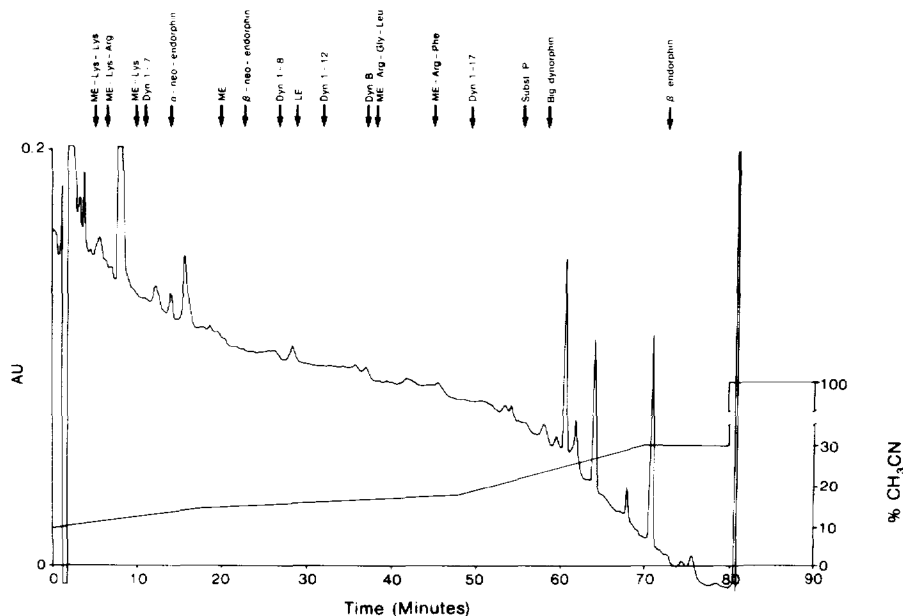


Fig. 3. Gradient RP-HPLC chromatogram of peptide-rich fraction from human tooth pulp. Conditions as in Fig. 1.

standard can not be considered to provide sufficient evidence to assign a molecular structure to the material eluting at this time without further structural elucidation.

In our analytical system, RP-HPLC of the peptide-rich fraction is used as a separation step followed by detection of the peptides of interest with several detectors. A combination of detectors is used because of the increased specificity obtained when several molecular parameters of endogenous peptides are monitored. Fig. 4 shows the tissue extraction and separation procedure and indicates the detection methods used for screening the HPLC fractions for endogenous neuropeptides and the MS techniques used to identify the individual peptides based on molecular structure.

2.3. Screening of HPLC fractions

The fractions collected after RP-HPLC gradient elution are screened for the presence of neuropeptides with several detection systems; each detector measures a different molecular parameter of the peptide. This combination of detection methods effectively increases the level of confidence in establishing the identity of the endogenous neuropeptides. RIA is used to measure peptides of interest based on their immunoreactivity. Commercially available RIA kits are used for quantification of ir-ME [45], ir-LE [46], and other ir-peptides of interest in the fractions collected from the gradient RP-HPLC (Fig. 2). These assays are performed on selected fractions collected during the chromatographic run at the elution time of the corresponding peptide standard.

RRA is used as a screen to detect all ra-peptides and is based on the competition between the endogenous peptide and a labeled ligand to bind to a

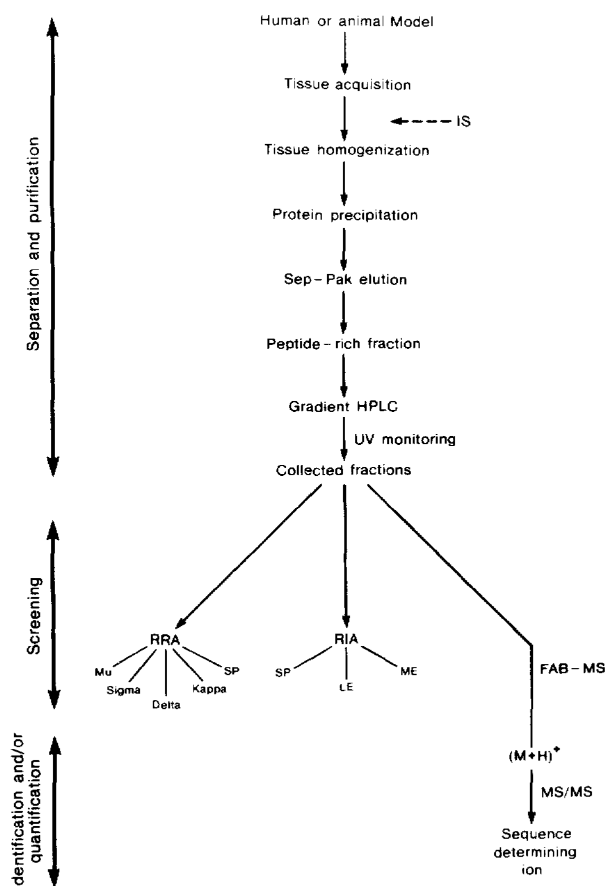


Fig. 4. Tissue extraction and analytical methodology for profiling and identifying neuropeptides. IS = internal standard.

receptor in a brain membrane preparation and permits the screening of entire peptide families and metabolites in tissue extracts [47—49]. By the judicious choice of appropriate competing labeled ligands and receptor populations, it is possible to detect the ra-opioid peptides derived from a given precursor in a tissue extract. The RRA sensitivity is not as high as that of RIA, but it offers a broader spectrum of specificity, a fact that makes RRA ideal as a screening method to identify those fractions containing opioid receptor-binding peptides. RRA can, in many cases, detect the presence of ra-peptides at the part per billion level (ng ra-peptide g^{-1} tissue).

A receptor-rich P2 or synaptosomal fraction [50] is obtained from a canine limbic system, and incubated at 37°C to release endogenous receptor ligands. Labeled ligand, which is purified first by HPLC before use, and unlabeled HPLC fractions are added to the synaptosomal membrane preparation and incubated (2.5 h, 0°C). Unbound label is rapidly and effectively removed by filtration with a glass fiber filter. The filter is washed several times to remove as much non-specific binding as possible, and the radioactivity remaining on the filter is measured. Non-specific (NS) binding is determined in the presence of

an excess of unlabeled peptide, and total (T) binding is determined in the absence of competing cold ligand. Specific binding (SB = T−NS) of each fraction provides semi-quantification of the ra-opioid activity of individual fractions when compared to the binding of known amounts of the opioid peptide ME with the same receptor preparation.

The RRA calibration curve is constructed from a set of known concentrations of cold ME ranging from 10^{-9} to 10^{-4} *M* and displays a sigmoidal relationship between the percent displacement of the tritiated ligand versus the molarity of the cold enkephalin. All quantification obtained from RRA is expressed as pmol ME equivalents when the calibration curve uses ME as the cold peptide. The data obtained by RRA represent the amount of a peptide present in an HPLC fraction, where that ra-peptide has the same affinity for the opioid receptors as does that quantity of ME.

Figs. 5 and 6 illustrate the data obtained by RRA of ra-peptides in human pituitary [29] and human tooth pulp extracts, respectively. The RRA data are superimposed on the gradient RP-HPLC chromatograms of these samples. The pituitary tissue shows a high level of receptor activity compared to the tooth pulp tissue; furthermore, the receptor activity in the pituitary is distributed across a wider range of hydrophobicity. This finding of numerous ra-peptides is in agreement with the fact that the pituitary gland is a synthesis and storage site for several protein and peptide hormones [51], whereas the tooth pulp is hypothesized to be an end-target tissue that might contain almost exclusively the shorter working peptides and their metabolites [30].

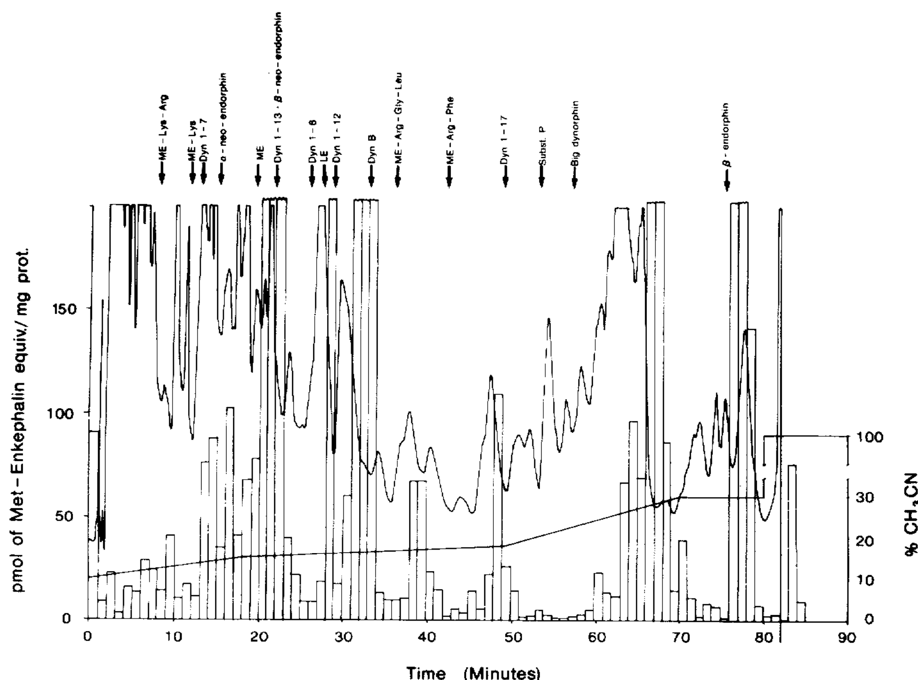


Fig. 5. RRA of gradient RP-HPLC fractions from peptide-rich fraction of human pituitary tissue. The bar graph represents receptor activity (ra) found in individual fractions expressed as pmol ME equivalents mg^{-1} protein.

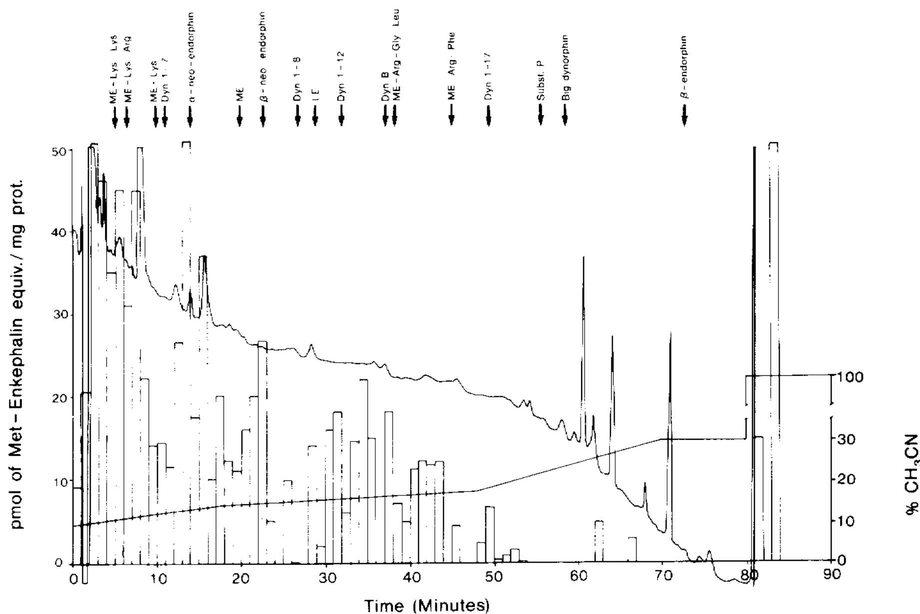


Fig. 6. RRA of gradient RP-HPLC fractions from the peptide-rich fraction of human tooth pulp. The bar graph represents receptor activity (ra) found in individual fractions and expressed as pmol ME equivalents mg^{-1} protein.

It should be noted that we usually observe high receptor activity in the first few HPLC fractions and also near fraction 85 (Figs. 5 and 6). This receptor activity may represent the additive contribution of unretained or very slightly retained molecules (very polar and/or small) in the first case, and of strongly retained molecules being eluted from the C_{18} column as a result of the organic modifier concentration reaching 100% in the latter case.

RRA provides semi-quantitative analytical data because the amounts indicated on the left-hand axis in Figs. 5 and 6 represent the amount of ME having the same binding characteristic in this particular receptor preparation as that of the ra-compound(s) in the given fraction. For a peptide that better competes with [^{3}H]etorphine for binding to the receptor than ME, the actual amount of the peptide in the tissue would be less than that indicated by RRA, whereas a peptide with less receptor affinity would be underestimated.

RRA has the analytical advantage of rapid and sensitive detection of opioid ra-endogenous peptides separated by gradient RP-HPLC and therefore it is an excellent screen for opioid activity in a biological tissue extract [25—29].

The authors highly recommend that any peptide or other compound, commercially available or synthesized in the laboratory, which is the target of extensive laboratory studies, be purified by HPLC before use. It is our experience that many laboratories simply assume for example that, when purchased, radiolabeled compounds are stable and pure, and remain so for an extended period of time. Our experience with several compounds, and especially the undecapeptide SP, does not support that assumption. For a radiolabeled material, it is simple to determine the RP-HPLC elution time of a larger amount of the cold compound, inject the radiolabeled material, collect only the labeled

compound eluting at the known retention time of the cold standard, and then use that HPLC-purified radiolabeled material in subsequent experiments. This purification process must be performed on a regular basis to monitor the chemical stability of the radiolabeled material. This purification is an important feature because, the higher the specific activities of these compounds, the greater the possibility that radiation damage will occur to that compound [52]. In addition, sulfur-containing amino acid residues frequently air-oxidize, a phenomenon that may not produce any experimental difficulties for cystine, but does for methionine [53] and SP.

2.4. *Mass spectrometric identification of endogenous peptides*

MS should be incorporated in the analytical scheme for the identification and quantification of endogenous neuropeptides because MS provides information on the most important analytical parameter, molecular structure [24, 25]. Of course, the limitation to providing an unambiguous structure for all experiments is the amount of material that can be extracted.

Once ir- and ra-peptides are separated by gradient RP-HPLC, structure elucidation is attempted to provide positive identification of the ir-/ra-active peptide. MS, particularly in the FAB mode, has the advantage of facile production of $(M+H)^+$ ions from peptides that have molecular weights up to 10 000 mass units [55], allows direct measurement of underivatized peptides, and possesses the most critical advantage of maximum molecular specificity by providing data in the high resolution-accurate mass-elemental composition mode from nanograms of compound. Furthermore, amino acid sequence-determining information [56] is obtained by using unimolecular decompositions, collision activated decompositions, and linked-field scanning methods [57].

2.4.1. *Protonated molecular ion $(M+H)^+$*

A FAB-produced $(M + H)^+$ ion readily provides the nominal mass of a given peptide in an HPLC-purified fraction and concomitantly increases our level of confidence concerning the structural identity of the ir- or ra-peptide in the HPLC fraction. For example, we have obtained $(M+H)^+$ ions at m/z 574 corresponding to the molecular ion of ME, and at m/z 556 corresponding to the molecular ion of LE, in the appropriate HPLC-purified fractions from a canine pituitary [58].

2.4.2. *Tandem mass spectrometry (MS—MS)*

Because a peptide containing n amino acids can have $n!$ different amino acid sequences, all with the same elemental composition and thus accurate molecular weight, one more structural elucidation step is needed to define the structure of the peptide. Tandem mass spectrometry (MS—MS) [59] effectively identifies a selected amino acid sequence-determining fragment ion in the mass spectrum of the peptide [60] (Fig. 7). MS—MS can be compared to other instrumental combinations such as liquid chromatography (LC)—MS and gas chromatography (GC)—MS. The first mass spectrometer (MS-I) serves as a purification step in a way similar to the role of gradient RP-HPLC described in

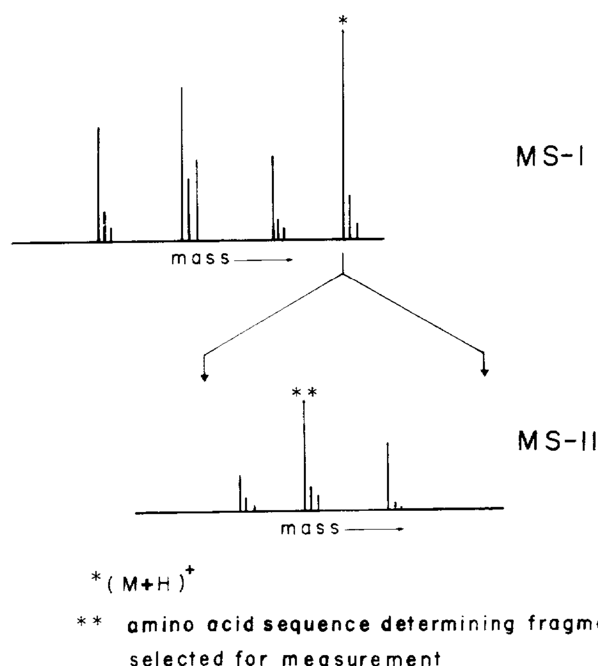


Fig. 7. Schematic representation of tandem mass spectrometry (MS—MS) for structural elucidation and quantification of peptides.

this chapter, and a second mass spectrometer (MS-II) provides the mass spectrum from one selected ion produced by MS-I [61]. For peptide identification, a unique amino acid sequence-determining fragment ion is then used for identifying and for quantifying the peptide present in the fraction [24].

For example, LE can be positively identified in the peak collected at the appropriate retention time by the presence of the C-terminal tripeptide fragment -glycine-phenylalanine-leucine (-GFL) in the mass spectrum of the $(M+H)^+$ ion of LE obtained by FAB-MS [40, 56, 62, 63].

Quantitative measurements of endogenous neuropeptides by MS can be achieved in as fast and facile manner with the use of stable isotope-incorporated peptide internal standard [40, 56, 60, 62, 63]. These internal standard compounds possess virtually the identical HPLC and MS properties as the endogenous peptides. Two ^{18}O-atoms are readily incorporated into the free carboxy group of the C-terminus of LE [40]. A known amount of this ^{18}O-containing LE is added to the tissue homogenate before the extraction procedure. As mentioned earlier, care must be taken to avoid back-exchange of ^{18}O with ^{16}O during the purification of the biological tissue extract.

Because LE incorporates two ^{18}O-atoms into only its carboxy terminus, the C-terminal tripeptide fragment ion -GFL (plus two hydrogens) originating from the added internal standard has a mass of 340, four mass units higher than the -GFL tripeptide (plus two hydrogens) from endogenous LE. The ion currents of the two -GFL fragments (exogenous, endogenous) are integrated separately by microcomputer techniques [64]. The ratio of these integrated ion currents, multiplied by the known amount of internal standard added to the tissue,

provides the most accurate measurement of the endogenous peptide in the original tissue extract, with a corresponding level of maximum molecular specificity and a sensitivity that generally ranges down to the ng level (pmol). Current developments indicate that femtomoles of peptides will soon be analyzed.

3. BIOMEDICAL APPLICATIONS

We use the analytical chromatography methodology described in this chapter to study the molecular basis of several disease states. Our research program aims towards monitoring several peptidergic pathways and individual peptides, and the metabolic relationships that exist between those pathways and peptides. The objective is to elucidate the molecular mechanisms involved in pain, stress, tumor formation, and some neurological disorders.

3.1. Neuropeptides and nociception

Opioid peptides are studied in human CSF obtained from chronic lower back pain patients who are potential candidates for surgery [36]. The three patient populations studied include those patients whose pain is relieved by a placebo, those who respond to spinal anesthesia, and finally those patients whose pain is not relieved (physiological non-responders) by this pharmacological treatment. We determined that placebo responders and physiological non-responders have low total ra-opioid concentrations, and physiological responders have a higher level of ra-peptides.

Tooth pulp tissue is very amenable to the study of opioid peptides and pain as discussed earlier. We demonstrated that ME decreases as one moves sequentially from the first tooth extracted to the fourth tooth extracted. Furthermore, we found that the concentration of ME decreases exponentially as a function of applied orthodontic force [30].

3.2. Neuropeptides and disease states

We screen human CSF from Alzheimer's [65] and amyelotrophic lateral sclerosis (ALS) patients in a study to detect peptidergic pathway abnormalities.

3.3. Neuropeptides in endocrine tumors

Pituitary tumors of various etiologies were screened and they all show opiate receptor activity. Normal pituitary tissue has a high level of opiate activity as illustrated in Fig. 6. Our data indicate that biosynthesis of opioid precursors is apparently operative in the tumor tissues studied and that the specific peptide processing enzymes in those tissues are also probably present in the tumor tissue [29].

4. CONCLUSIONS

Many of the recent developments in neuropeptide research can be attributed

directly to experimental developments and refinements in RP-HPLC methodology, an extremely useful chromatographic technique that permits the effective separation of endogenous peptides from a biologic tissue with high resolution, high speed, and high recovery.

HPLC is an integral part in all aspects of our research. Although it is true that we have attempted to show that HPLC can not and should not be depended upon exclusively to purify a selected peak from all other potential peaks present in a biological mixture, HPLC does play an extremely useful role in several other experimental respects: Sep-Pak preparation of a peptide-rich fraction; high resolution of endogenous peptides; high recovery; effective gradient separation of peptides possessing a wide range of hydrophobicities; efficient purification of radiolabeled and cold peptide standards and ligands; and accurate and reproducible determination of the retention times of peptide standards. Much of the RIA, RRA, and MS developments discussed in this review could not proceed as far as described here without RP-HPLC analytical methodologies.

A combination of several specific analytical methods such as gradient RP-HPLC, RIA, RRA, and FAB-MS is used effectively to assign an opioid activity profile to a biological tissue extract [24, 25, 57]. With this combined methodology, we can screen for the presence of endogenous ra- or ir-peptides present in a biological extract at the nanogram level, corresponding to the pmol g^{-1} tissue level of sensitivity for the smaller peptides.

Molecular specificity is low with UV detection, increases through RRA, RIA, and FAB-MS-$(M+H)^+$, and is maximized with FAB-MS—MS. MS—MS provides a unique amino acid sequence-determining fragment ion, and quantification is achieved by using a stable isotope-incorporated peptide internal standard.

5. SUMMARY

Biological tissues and fluids are subjected to gradient reversed-phase high-performance liquid chromatography (RP-HPLC) separation and the neuropeptide profile of the collected fractions is obtained by radioreceptorassay (RRA) using a broad-based competing ligand. Radioimmunoassay (RIA) is also used to detect specific neuropeptides in the HPLC-purified fractions. Further confirmation of the identity of the peptides present in the tissue is obtained by mass spectrometry (MS) in the fast atom bombardment (FAB) mode. FAB-MS produces the protonated molecular ion of the peptide and allows direct measurement of underivatized peptides at the nanogram level, with increased molecular specificity. FAB-MS—MS identifies a unique amino acid sequence-determining ion in the mass spectrum of a peptide and offers maximum molecular specificity. This analytical chromatography methodology is applied to the study of the molecular basis of several disease states by monitoring several peptidergic pathways and individual peptides and their metabolic relationships. Molecular mechanisms involved in pain, stress, tumor formation, and neurological studies are studied.

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