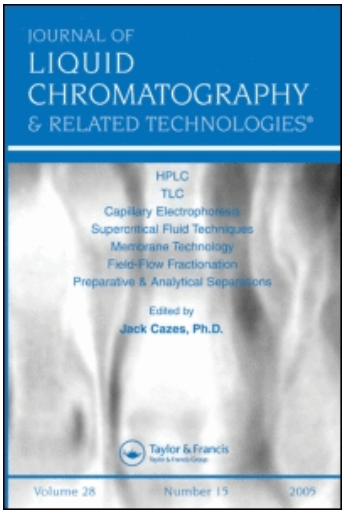

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Isolation, Separation, and Detection of Enkephalins: A Review of Methods for High Performance Liquid Chromatography and Capillary Electrophoresis

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ISOLATION, SEPARATION, AND DETECTION OF ENKEPHALINS: A REVIEW OF METHODS FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS

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ABSTRACT

High Performance Liquid Chromatography(HPLC) and Capillary Electrophoresis (CE) are valuable analytical techniques for the determination of neuropeptides in biological fluids. A crucial aspect of HPLC and CE analyses is the preparation of samples. This review outlines sample preparation protocols for HPLC and CE methods that have been used for this determination, with a focus on enkephalins. Sample preparation protocols have ranged from simple techniques of extraction and centrifugation to solid phase extraction (SPE) and ultrafiltration. Additionally, this review also discusses the final determinant steps of HPLC and CE using various modes of detection . Detection modes for the HPLC methods included ultraviolet (UV), fluorometry, electrochemical and mass spectrometry (MS). Modes of detection when using CE for this determination have been more limited, focusing on UV and MS detection modes.

Introduction

Since the discovery of the pentapeptides, methionine enkephalin (ME) and leucine enkephalin (LE) by Hughes in 1975 (1) there has been a great deal of interest focused not only on the function of these compounds and other members of the opioid class but also on selective and sensitive methods to allow for their measurement in biological systems (2). A search of the

literature conducted with a time frame from January 1990 to January 1995 indicated more than 1500 citations on enkephalins with a substantial number of these reports concerned with analysis. Much of the activity in the determination of these compounds has centered on the use of radioimmunoassys (RIA) which, for some time, was the only technique that afforded sufficient sensitivity to be of use to researchers. In RIA, antiserum generated to peptide and protein conjugates is generally directed towards the C-terminus. Attack at the C-terminus of the molecule appears to afford better selectivity when compared to attack at the N-terminus of the molecule. However, since many of the enkephalins in questions are structurally similar, a certain amount of cross-reactivity between related peptides would be expected. (3).

In recent years, developments in high performance liquid chromatography (HPLC) and more recently, capillary electrophoresis (CE) have allowed for their use in neuropeptide analysis. This review will concentrate on HPLC and CE methods for the determination of ME and LE. It will outline methods of sample preparation for this class of compounds and also provide selected HPLC and CE methods that have been used for the determination of these substances. Methods of sample preparation and analysis outlined in this review range from very direct protocols that may be appropriate for some samples to extremely intricate methods that have been used for other sample types. Comprehension of this information should permit a scientist to make informed decisions regarding the assessment of neuropeptides in biological samples.

Sample Preparation

Before any analysis can proceed, some form of sample preparation must be performed. With some compounds and samples this can be as simple as dilution and filtration prior to analysis. Alternatively, it can be a multi-step sample preparation protocol. In the case of neuropeptides, initial efforts utilized the unit operations of solubilization, centrifugation, and

filtration in a variety of combinations. Loeber (4) extracted rat pituitary glands with a mixture of 0.2 M HCl and cold acetone. After the samples were homogenized, they were centrifuged at 30,000 g and the supernatant removed. The supernatant was lyophilized and the sample dissolved in ammonium acetate at a pH of 4.15 prior to HPLC analysis. Mousa and Couri (5) analyzed serum for enkephalins and neuropeptides using a sample preparation technique which entailed centrifugation at 2400 g prior to analysis. A third method (6) reported for the extraction of enkephalins in tissue samples was the use of a mixture of acetic acid, HCl and beta-mercaptoethanol which contained pepstatin and phenylmethyl sulfonyl chloride. After extraction, the samples were centrifuged at 26,000 g followed by open column chromatography on Sephadex G-10 or G-25, or precipitation with TCA. Samples treated with TCA were centrifuged at 26,000 g and the compounds of interest extracted with ether prior to HPLC analysis.

Laatikainen and his coauthors (7) reported a method for blood samples where they were collected in polyethylene tubes containing heparin and aprotinin. Samples were then chilled and centrifuged at 1000 g for 10 minutes at O degrees C. After addition of an internal standard, plasma proteins were precipitated by treatment with 0.1 M acetic acid and acetonitrile. The samples were centrifuged at 4500 g and diluted with distilled water before analysis with cation exchange HPLC.

A second type of sample preparation that has been used in this assay is ultrafiltration (UF), a method used in the HPLC analysis of ME in the retina of the developing rat (8). In this technique, samples were prepared for analysis by mixing in a phosphate buffer and ultrafiltration through a 10,000 MW cutoff membrane. UF also has been employed extensively in the analysis of numerous pharmaceutical compounds and for the study of protein bound and unbound drugs. (9,10) . The sample preparation can be performed with either a centrifuge or a syringe (11, 12).

Solid phase extraction (SPE) is a third method of sample preparation and has replaced many of the earlier protocols . There is substantial literature pertaining to the basics of this technique (13), and many vendors of these columns have developed data bases for applications (14,15). The SPE technique for neuropeptides has been pioneered by Desiderio (16). As an example of this SPE protocol , tissue was homogenized with 1N acetic acid and the proteins precipitated with a mixture of acetone/ 0.1N HCl (8/2). The resulting solution was centrifuged at 27,000 g for 20 minutes. The supernatant was withdrawn, dried under a stream of nitrogen, resuspended in 1% trifluoroacetic acid (TFA), and applied to a previously conditioned C18 solid phase extraction column. The salts were removed by a TFA wash and the peptide rich fraction eluted with a mixture of acetonitrile/0.05% TFA (8/2) for HPLC analysis (17). Figure 1 provides an outline of the overall scheme that was employed.

SPE was applied by Fleming and Reynolds for the determination of ME and LE in the rat brain (18) . Since the final analysis used electrochemical detection for the HPLC system and was operated at high applied potentials it was necessary to develop highly effective sample preparation protocols. Fleming and Reynolds utilized a technique called chromatographic mode sequencing (CMS) which resulted in decreased amounts of electroactive interfering substances. In this method, samples were initially extracted with acetic acid , lyophilized and frozen at -70 degrees C. The lyophilized samples were resuspended in 10% TCA containing 0.1% sodium metabisulfite, and centrifuged. The samples were extracted with two volumes of diethyl ether and vortexed. The ether layer was removed and nitrogen blown over the remaining aqueous layer to eliminate any residual ether. The pH of this layer was adjusted to approximately 2 with 1.0 M NaOH and phosphate buffer (pH 2.3) for a final phosphate concentration of 40 mM. An aliquot of tissue no larger than 300 mg was applied to a 400 mg C-8 SPE column. The column was washed with 3.0 ml of 50 mM potassium dihydrogen phosphate (pH 2.3), 2.0 ml of water/methanol (83/17 v/v), 2.0 ml of water /acetonitrile (92/8 v/v) and 2.0 ml of water/acetic

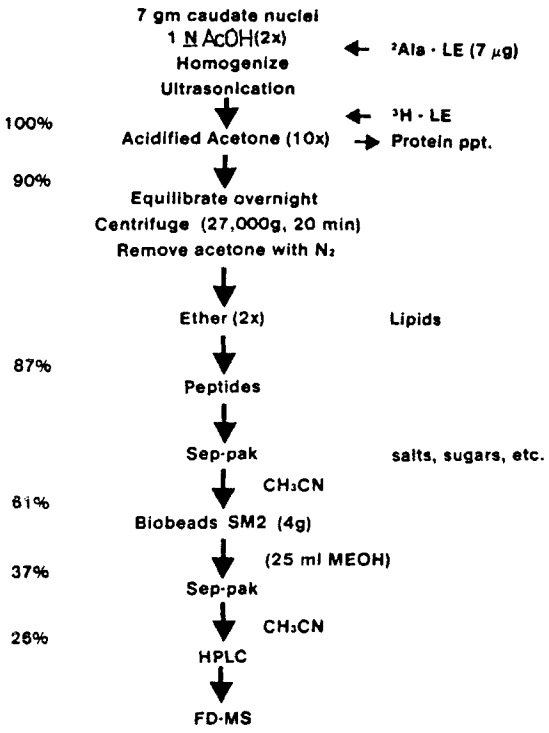

Figure 1

Solid Phase Extraction Protocol (59)

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acid (85/15 v/v) with a 3.0 ml water wash used between each organic wash step. The enkephalin fraction was eluted with 1.0 ml of triethylamine phosphate/acetonitrile (50/50) . The extract was dried under a stream of nitrogen and redissolved in water. It was applied to a 180 mg activated Type W column. This column was washed with 3.0 ml of water, 2.0 ml of the previous water/methanol mixture, 2.0 ml of the water/acetonitrile mixture and 2.0 ml of water/acetic acid (88/12 v/v). A water wash was used between each organic wash. The enkephalins were eluted with acetonitrile/water (56/44 v/v), evaporated to dryness and then redissolved in an appropriate

Table 1

Additional Uses of SPE for the Preparation of ME and LE

Compounds Analyzed	Reference
ME, LE	21, 26
Neuropeptides including ME	22, 23, 24, 25, 27

amount of the 50 mM phosphate buffer for analysis. While this was an extremely detailed protocol, the authors indicated that no spurious peaks appeared, and that after injecting these tissue extracts for a week, it was only necessary to wash the column with 100% methanol before additional analyses.

In the determination of beta-endorphin fragments in human plasma (19), an acidified sample was applied to an C18 SPE column. which had been conditioned with methanol, 6M urea and water. The acidified plasma was applied to the column and washed with water and 4% acetic acid. The retained peptides were eluted with a mixture of 1-propanol/acetic acid (96/4). While this method focused on endorphins, alteration of the elution solvent would make it appropriate for ME and LE.

Additional uses of SPE cartridges have allowed for the development of rapid isolation protocols for enkephalins and endorphins. In this method (20), C-18 SPE columns were conditioned with methanol, water and 0.5 M formic acid-pyridine at pH 3 or pH 4. Enkephalins were isolated using the pH 3 system while endorphins were isolated with the pH 4 solvent system. A number of other SPE protocols have been reported for enkephalins and are summarized in Table 1.

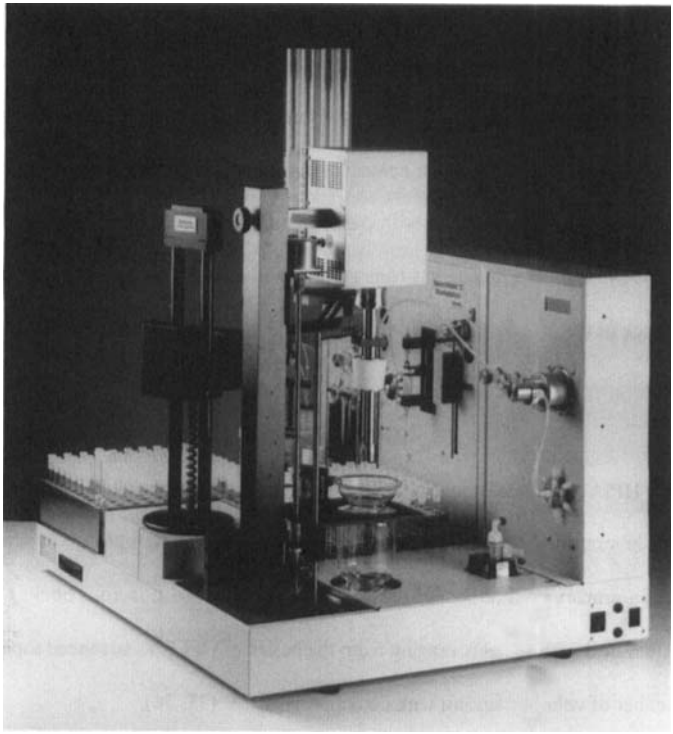

Figure 2

Automated Sample Preparation Workstation

An exciting development in the use of SPE for sample preparation of ME and LE is the commercialization of a number of automated systems for solid phase extraction (28). These systems range from a full fledged laboratory robot to a robotic workstation. An example of one of these instruments is seen in Figure 2. While no one has yet reported the use of these systems for these isolations, one could expect that the uses of automated systems will increase in the clinical laboratory as prices decrease and the demand for determinations continues to rise.

Another mode of sample preparation for enkephalins is microdialysis. Microdialysis is a very attractive technique since it can be used on freely moving conscious

animals (29). Microdialysis can introduce and remove molecules from the brain and the sample will contain no blood cells or macromolecules. Samples obtained by this technique can often be used without the need for additional sample refinement. One of the concerns about samples prepared in this fashion is that they must contain sufficient amounts of the compound of interest to detect. However, salts which dialyze with the compound of interest may need to be removed prior to analysis (30). This technique has been applied to the determination of neuropeptides in median eminence of the ewe by Advis and Guzman (31).

HPLC Analysis

HPLC is widely used in analytical and neuroscience laboratories in two distinct ways. In some laboratories it is used as a supplement to established RIA protocols and in others it is employed as a primary method for enkephalin analysis. There are numerous books about HPLC (32, 33, 34) that deal with subjects ranging from the basics of HPLC to advanced topics. There also are a number of volumes dealing with detection in HPLC (35, 36).

Five modes of detection have been used in the HPLC analysis of the enkephalins; spectrophotometric, fluorescence, electrochemical, radioimmunoassay (RIA) and mass spectrometry (MS). This review will briefly discuss all of these modes of detection except RIA.

The first type of HPLC detection system for enkephalins is ultraviolet spectroscopy (UV). Most peptides exhibit no characteristic chromophore, so detection in the 200 nm region is used. Figures 3 and 4 provide illustrations of this mode of detection showing the separation of ME and LE on a reversed phase HPLC with a mobile phase of acetonitrile/water and TFA with detection at 200 nm. Since many solvents associated with HPLC adsorb in this region some compromise is seen between sensitivity and wavelength. While this region can be used for the detection of these neurochemicals, a substantial number of other compounds also absorb in this region. ME and LE contain tyrosine residues which absorb in the 254 and 280 nm

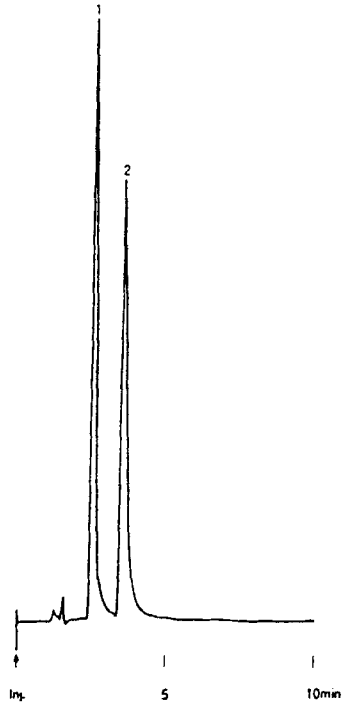

Figure 3

Reversed Phase Separation of ME and LE (59)

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region, so this wavelength can be used for these compounds but at a loss of sensitivity compared to detection at 200 nm (37). Initially, HPLC detectors had fixed wavelengths of either 254 or 280 nm, so these wavelengths were the obvious choice. As detector technology evolved, variable wavelength detectors were developed that allowed the analyst to monitor the 200 nm region of the spectra with good signal to noise (S/N) ratios. As detectors continued their evolution, there have been two developments in the technology: scanning and photodiode array (PDA) detectors. The scanning detectors scan peaks of interest as they elute from the HPLC column while the PDA detector affords additional capabilities such as absorbance ratioing for peak purity, searching of

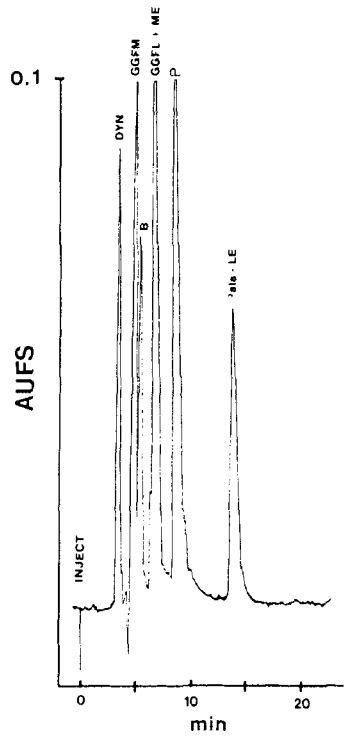

Figure 4

Reversed Phase Separation of Neuropeptides

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spectral libraries, and the calculation of derivative spectra. Since many similar compounds have similar UV spectra, the ability to calculate and display derivative spectra is an important component of a detection system. PDA detection systems provide an excellent complement to MS detection.

Fluorescent and other detection mechanisms has also been applied to the analysis of enkephalins. A paper by Zhang (38) reported the determination of ME, LE and substance P using fluorescence detection of the 9-fluorenylmethyl chloroformate derivative (FMOC). The

derivatized compounds are separated in a two step gradient in approximately 18 minutes with lower limits in the range of 300-500 fmoles. After treatment of the FMOC-neuropeptide derivative with mild acid, the intact neuropeptide could be recovered. Jencen (39) reported on the formation of napthalene-2,3-dicarboxyaldehyde/cyanide (NDA/CN) derivatives of ME, LE and D-[Ala^5] -ME using conventional fluorescence detection with column switching for increased chromatographic selectivity. This work was expanded to allow for chemiluminescence detection. Electrochemical detection also has been used for the determination of neurochemicals with numerous references appearing in the literature (40). While other modes of detection for HPLC are valuable for the analysis of neuropeptides, none has the broad applicability or provides the amount of information that is available from mass spectrometry (MS). Research in liquid chromatography- mass spectrometry (LC-MS) has been active since the 1960's. There has been an expansive evolution of the technology in the 1980's, with this technology expanded to the analysis of neuropeptides. An overview of LC-MS has been provided by Tomer and Parker (41), while the recent article by Heath and Giordani (42) provides a comparison of UV, fluorescence and electrospray for the detection of selected peptides.

In 1982, Yamada and Desiderio (43) published a method for the analysis of endogenous LE using a combination of HPLC and Field Desorption MS (FD-MS) . In this application samples were prepared off-line for analysis. Kenyon and coworkers reported the sequencing of underivatized peptides by Direct Liquid Inlet (DLI) LC-MS using a reversed phase column and a buffered mobile phase consisting of triethylammonium acetate and acetonitrile (44) . Positive and negative ion spectra were obtained and M+1 and M-1 ion were seen for the compounds of interest. The use of thermospray (TSP) LC-MS in neurochemistry was reported in 1987 by Artigas and Gelpi (45) who provided data of the MS of 19 indolic compounds in positive and negative ion modes. In the past few years, flow fast atom bombardment (FAB) MS has been used extensively for the identification of selected neuropeptides. FAB allows for the production of

M+H ions from peptides up to 10,000 daltons for the determination of underivatized peptides (46) . An article by Hill (47) stated that the two developments in bioanalytical mass spectrometry that allowed it to develop at an accelerated pace were tandem MS (MS-MS) and spray ionization. MS-MS enhanced the signal to noise ratio and allowed for better sensitivity and spray ionization allowed MS to be applied to molecules with a high mass and low volatility such as proteins and peptides. An extreme amount of activity in the use of electrospray ionization (ESI) and it's variants has been reported. Electrospray is another soft ionization technique (48) like thermospray, but unlike thermospray allows for the production of multiply charged molecular ions. It has been successfully applied to assays by a number of researchers. Dass and colleagues (49) reported the use of ESI-MS for the analysis of opioid peptides and the quantification of endogenous ME and beta-endorphin. ME and LE produced only [M + H] + ions while the other peptides produced a series of multiply charged ions. Samples of pituitary extract were prepared using a SPE protocol and ME was quantified with an internal standard. The ME content in the extracts ranged from 4.7 to 9.1 pmol/mg protein. Dimond (50) has provided a technical review of mass spectrometry for biomolecular analysis and states that ESI-MS produces molecular weight data with an accuracy of 0.01% for proteins and peptides in the range of 100-100,000 daltons and can be performed on 1 pmol of material. Figure 5 gives an example of the spectra recorded for 6 neuropeptides . In this application, a fused silica column 50 um ID x 8 cm was packed with 10 um C-18 material. A gradient elution was performed using a mobile phase of 2-50% methanol in 0.25% acetic acid at a flow rate of 0.82 ul/min. Nanoscale packed column capillary LC coupled to continuous flow FAB has been applied to a number of peptides including synthetic mixtures of bioactive peptides (51) . The column consisted of fused silica with ID of 50-74 um divided into 2.5 m lengths and packed with 780 um C-18 material. The mobile phases consisted of linear gradients of acetonitrile/water or methanol/water containing 0.1% TFA. Figure 5 provides a sample separation of seven neuropeptides using this technique. Woolfit (52) reported the LC/MS

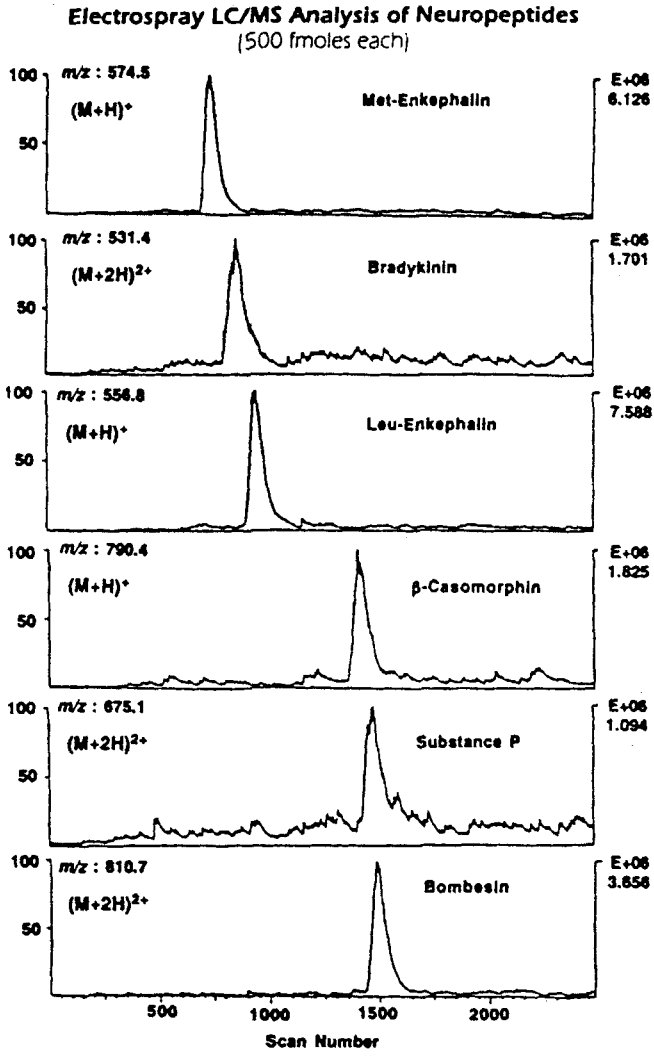

Figure 5

Series of Electrospray SIC of a Mixture of Neuropeptides (50)

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analysis of an enkephalin mixture using a liquid secondary ion MS interface and Lovelace (53) has described the determination of ME in human pituitary tissue by multidimensional reversed phase HPLC, radioreceptor assays, FAB-MS and MS-MS. A paper by Kusimerz (54) outlined a FAB-MS method for the quantitation of picomole levels of ME in human pituitary extracts using a deuterated internal standard followed by multidimensional LC. The application of pattern recognition techniques to MS data for sequencing C-terminal peptide residues was described by Degoda and Pulfer (55) . In this application, pattern recognition techniques were investigated on sequence information obtained from FAB and MS-MS data and applied to amino acid sequences up to pentapeptides. Recently, Emmett and Caproli reported a micro-electrospray technique for the high sensitivity analysis of peptides and proteins (56). Figure 6 shows injections of ME desorbed from a C-18 packed spray needle with (a) 10 ul of 5 fmol/ul in Ringers solution and (b) 10 ul of 100 amol/ul in water. Table 2 provides additional references on the analysis of enkephalins and other selected neuropeptides by HPLC.

Reversed phase HPLC columns are utilized for peptide determinations since they have excellent resolution and are able to resolve similar polypeptides which might differ by only one amino acid residue. Other developments in reversed phase HPLC variants involve the application of a larger pore size particle which allows larger peptides better access to the interior of the silica and also the development of a C-4, butyl phase. The physical dimensions of HPLC columns have been altered with narrow bore columns having a diameter of 2.1 mm that allow for increased sensitivity and flow rates of about 20% of standard 4.0 mm diameter columns. Minibore columns with 1.0 mm diameter offer a five fold improvement in sensitivity and flow rates in the 25-50 ul /minute flow range. Finally, there have been a resurgence in capillary chromatography where flow rates are in the 1-5 ul/minute flow range. Review articles and several other books dedicated to the determination of proteins and peptides by HPLC should be consulted for further information (62-64).

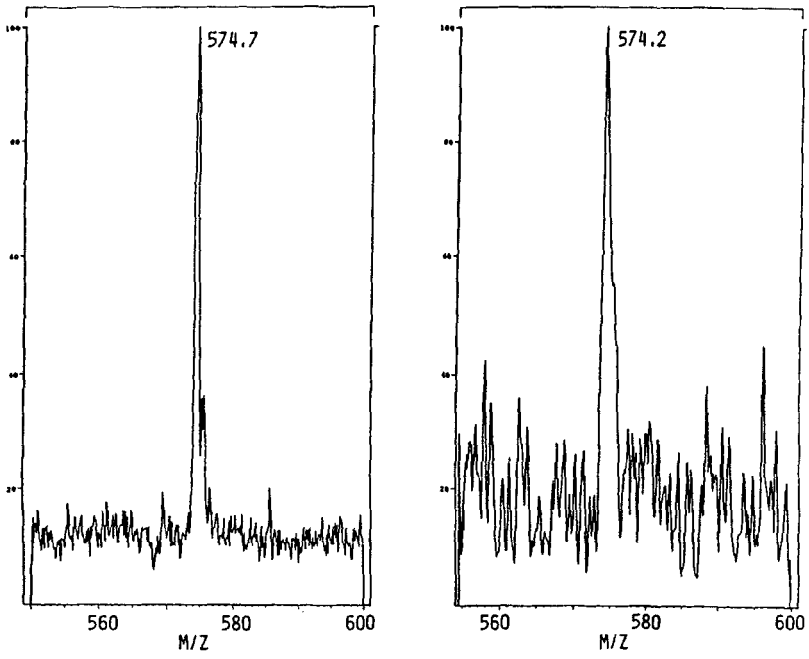

Figure 6

Micro-ES Spectrum of ME Desorbed from C-18 Packed Spray Needle (56)

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Table 2

Additional HPLC Methods for the Determination of ME and LE

Column	Detection	Reference
μBondapak C18	UV/RIA	57
C18	UV	58
μBondapak C18	RIA	59
C18	Electrochemical	60
μBondapak C18	UV	61

Capillary Electrophoresis

HPLC, with it's various modes of separation and detection, has provided the neuroanalyst with excellent capabilities. The introduction of CE in the 1980's, however, has provided exciting capabilities for the analysis of neuropeptides. CE offered rapid analysis times, high column efficiencies, high resolution, and automation in both methods development and analytical phases of the determination (65). It is not within the scope of this manuscript to provide an extensive review of this technology in it's various forms, and the reader is referred to other reference materials including some recent reviews and books on the subject (66 -69).

HPLC offers a wide variety of modes of detection for the analysis of ME and LE. Presently CE has limited detection options. The most recent generation of commercially available instruments come equipped with a UV detector. These detectors vary greatly with some vendors providing a variable wavelength detector while others offer variable wavelength detectors with scanning capability. Additionally, CE instruments equipped with photodiode array (PDA) detectors are becoming common. Even though CE uses very small volumes of sample and buffer, it cannot be called a trace technique, so a number of protocols and instrumental variations have evolved to allow for the enhancement of sensitivity (70). These embellishments have occurred essentially in two areas: sample concentration and cell geometry. One sample concentration strategy has been stacking which is based on the electrophoretic concentration of the peptide of interest and has been said lower the detection limits of compound 5-fold (71). One vendor now markets a special capillary that contains a small amount of reversed phase HPLC packing at the injection site, thereby allowing for enhanced sensitivity. In the area of cell geometry, there have been developments of bubble cells and Z-shaped detector optics (72). One vendor has introduced a high sensitivity option to allow for enhanced UV detection (73). Fluorescence detection is also becoming commercialized with vendors offering laser induced fluorescence (LIF) and a filter based instrument fluorescence detector. In the examination of peptides, UV detection at 200 or

280 nm has been used for the same reasons that it is employed in HPLC determinations. In CE, the capillary column becomes the detector window and on-column detection of approximately 100 pg has been reported (74). Moreover, with LIF, attomole detection levels of selected compounds have been published (75). In addition to these two modes of detection, indirect UV and amperometry have also been employed in CE (76, 77).

While the most widely used detection mode in CE is UV, considerable activity has been associated with the coupling of CE with MS. A review of CE-MS was recently published by Niessen and coworkers (78). Mosley and others (79) reported the use of CE-MS with continuous flow FAB interface for the determination of bioactive peptides. ESI has also been applied to this determination. CE-ESI-MS has also been applied to the analysis of small proteins up to 30,000 daltons. Muck and Henion (80) reported the determination of ME and LE in equine CSF by microbore HPLC and CE-MS. The CE separation was performed on a 90-100 cm x 100 um I.D. untreated fused silica capillary using a 50/50 mixture of acetonitrile and 20-30 mM ammonium acetate buffer @ pH 6.8 with MS detection.

At the 1994 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Sweedler (81) presented a paper on the analysis of neuropeptides using multichannel detection in CE. While it was not applied to the enkephalins, laser-induced fluorescence and radiochemical detection was applied to the determination of eight neuropeptides from the giant marine snail with the aim of measuring these compounds within the processes of the neurons and following the release of the peptides from different presynaptic release sites. The detection system was an imaging spectrograph and a slow-scan CCD detector. The system was able to detect zeptomole (10^{-21}) amounts of fluorescent labeled peptides from a nanoliter sample.

The applications base on protein and peptide analysis continues to grow at a rapid rate as can be seen in Table 3, and the use of specialty columns for capillary gel electrophoresis and other modes of separation will increase. There are now kits available from vendors that will

Table 3

Selected Applications of CE for the Determination of Proteins and Peptides

Protein	Reference
Human Growth Hormone	82
Anticoagulant Peptide	83
Growth Hormone Releasing Peptide	84
Motilin Fragments	85
Tryptic Peptides from Myoglobin	86

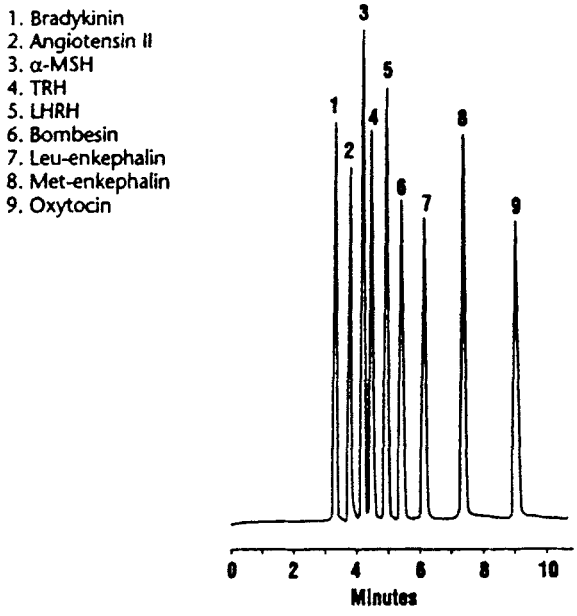

Figure 7

Sample Electropherogram of Peptides Using SEC Separation

(Compliments of Bio-Rad)

Table 4

Selected HPLC and CE Techniques for Amino Acid Composition

Derivative	Reference
PITC	88
OPA	89
FMOC	90
Dabsyl	92

allow many of these separations to move from research environments to routine usage. Figure 7 illustrates the separation of a peptide standard mix using one of these kits. With the extreme interest in the assay discussed in this review, one would expect the applications base for neurochemicals and neuropeptides to continue evolving at a rapid pace.

Confirmatory Techniques

MS, in one of its many forms, is the most direct method to confirm the identity since it can provide not only extremely precise information about the molecular weight of a substance but also provide information about it's composition due to the fragmentation pattern of the material of interest.

Should one not have access to an MS, a series of studies using amino acid analysis could be performed to further identify a substance. Amino acid analysis with HPLC or CE using any one of the accepted techniques listed in Table 4 would provide information regarding the amino acid composition of the substance. In the case of ME and LE, the resulting information would prove that each was a pentapeptide but would not address the amino acid sequence . To determine the sequence of the amino acids, an Edman degradation

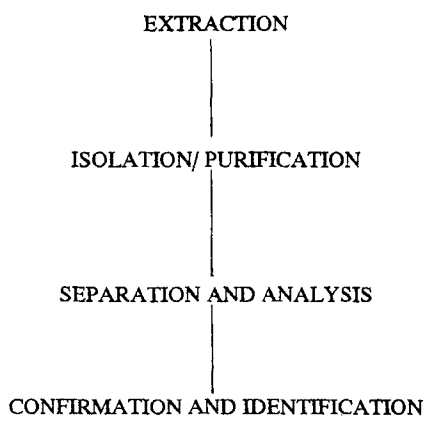

Figure 8

General Protocol for the Isolation and Identification of Neuropeptides

(87) would be performed. The Edman degradation is a classical method that can be accomplished in either a manual or automated fashion. These complimentary techniques could be used to provide more information about the peptide in question.

The determination of the enkephalins ME and LE in biological fluids is a multi-step effort requiring extraction, isolation, separation and final identification. Figure 8 provides an overview of the general protocol needed to successfully isolate and identify ME and LE .

Conclusion

This review has discussed previous and current applications on the isolation, separation and analysis of enkephalins. It also provided the scientist with a bibliography to seek further information since reseach efforts indicate that neuropeptides are not only neuromodulators and neurotransmitters, but also growth factors, and methods of analysis of these compounds are becoming increasingly important.

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