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MEASUREMENT OF ENDOGENOUS LEUCINE ENKEPHALIN IN CANINE THALAMUS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FIELD DESORPTION MASS SPECTROMETRY

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SUMMARY

A combination of high-performance liquid chromatography and field desorption mass spectrometry is used to quantify endogenous amounts of leucine enkephalin in canine thalamus tissue. Reversed-phase high-performance liquid chromatography effects rapid high resolution of brain neuropeptides using a triethylamine formate buffer. An internal standard, ^{2}Ala-leucine-enkephalin, is used. Field desorption mass spectra of neuropeptides generally display only protonated molecular ions. $(M+H)^+$ ion currents of endogenous leucine enkephalin and internal standard were integrated by field desorption mass spectral-selected ion monitoring techniques. The ratio of the two integrated ion currents was used to calculate endogenous amount of leu-enkephalin in thalamus tissue extracts. Leucine enkephalin was determined in this structurally unambiguous fashion in canine thalamus tissue at 50 ng/g thalamus tissue, or the 50 part per billion level.

INTRODUCTION

The objective of this research is to quantify in a structurally unambiguous manner the endogenous amount of an opioid peptide, leucine enkephalin (LE), in a selected canine brain region, the thalamus. This objective is accomplished with a combination of two instrumental methods, high-performance liquid chromatography (HPLC) and field desorption mass spectrometry (FD-MS).

The long-term objective of this laboratory is two-pronged: utilize mass spectrometry in elucidating the structure of unknown peptides[1] and measurement of endogenous amounts of bioactive peptides in biologic tissue[2]. Use of FD, chemical ionization (CI), electron ionization (EI), fast atom bombardment (FAB), and any other appropriate mass spectral ionization technique followed by collision activation (CA), link-scanning (*e.g.*, B/E), and any other analysis technique to produce sequence ions is the mechanism to obtain the amino acid sequence of an unknown peptide. Once the structure of the biologically active peptide is elucidated, the second phase is to quantify, with optimal structural specificity, endogenous amounts of this peptide in biologic fluids and tissues. Biologic matrix effects demand an internal standard for

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quantification of each peptide and metabolite of interest. Biologic matrix effects are ill-defined, but are the sum of peptide–protein, –lipid, –saccharide plus other unknown interactions that cause the observed difference between measurement of a pure solution and a biologic extract.

Whenever endogenous amounts of neuropeptides are measured by utilizing fluorescence, electrochemical, ultraviolet, or other detection methods following HPLC separation, then that one parameter (fluorescence quantum yield, half-wave potential of an electroactive species, UV absorbance, etc.) and not structure is determined at that specific HPLC retention time. On the other hand, many laboratories utilize radioimmunoassay (RIA) for quantification[3] of neuropeptides because of facile production of antibodies to a neuropeptide, short measurement time, low cost per measurement, and high sensitivity. An objective of RIA measurement is to have total molecular specificity so that chromatographic separation is not necessary. This criterion has not always been met[4,5]. The presence of metabolic enzymic apparatus for rapid and massive release of opioid peptides during certain metabolic events (death[6], pain, euphoria, etc.) implies ready availability of precursor molecules[7]. On the other hand, the extremely rapid metabolism of potent endogenous opioid peptides that is required in neural events implies the presence of metabolizing enzymes with high turnover number. Both metabolic situations provide precursors and metabolites which may have indistingishable RIA activity *vis-a-vis* the bioactive neuropeptide. RIA measurements of somatostatin, Leu-enkephalin, and bradykinin demonstrate this point. A molecular parameter is needed (mass of molecule or specific sequence-related ion) for quantification of a particular neuropeptide in a complex biologic extract.

This laboratory has developed volatile HPLC buffer systems[8], internal standards, extraction schemes, methods to avoid catabolism and metabolism, and FD-MS measurements of neuropeptides[8] in biologic tissue including canine caudate nucleus[9], hypothalamus[10], and tooth pulp[11]. Triethylamine–formate (TEAF) has been used as the HPLC buffer to quantify femtomole (fmol) amounts of somatostatin[8]. Picomole amounts of synthetic Leu-enkephalin solutions were quantified by FD-MS[12]. No other laboratory has reported on quantifying underivatized endogenous peptides by FD-MS.

MATERIALS AND METHODS

Tissue procurement

Dogs under pentobarbital anesthesia are exsanguinated via a femoral artery. The cranium is opened and brain removed. Caudate nuclei, hippocampus, pituitary, cerebellum, hypothalamus, cortex, spinal cord, olfactorum tubercle, pons, thalamus, etc. are excised and placed into liquid nitrogen within 4 min. Samples are stored at −70°C. Tissue weights are wet weight.

Internal standard and spiking

A 200-μg amount of internal standard (^{2}Ala-LE) (Bachem, Torrance, CA, U.S.A.) per gram of tissue in 20 ml Tris buffer (pH 7.4) is added to the homogenization flask. A 200–250 ng "spike" of LE is added, as indicated below (Fig. 2).

Sample preparation

Samples (2 g) are defrosted, mixed with 3 ml of 1.0 *M* acetic acid and homogenized for 3 min at 0°C with a VirTis 23 (Gardiner, New York, NY, U.S.A.) or Polytron homogenizer. This pH disrupts any peptide tissue protein complexes. Cell membranes in this solution are disrupted with a Kontes (Evanston, IL, U.S.A.) ultrasonic generator (300 W, 3 min) with a 4.5-in. probe.

Tracer

Homogenate is diluted ten-fold with either 1.0 *M* acetic acid or acetone–0.01 *M* HCl and transferred to a polypropylene centrifuge tube. Tritiated LE (tyrosyl-[3,5-^{3}H]Gly_2-Phe-Leu) (0.1 μCi, New England Nuclear, Cambridge, MA, U.S.A.) is added. Samples equilibrate overnight at 4°C[13].

Protein precipitation

Proteins are precipitated with ten volumes of acetone–0.01 *M* HCl (80:20) and removed after centrifugation at 15,000 *g* for 20 min at 0–5°C (Beckman J-21, Palo Alto, CA, U.S.A.). Supernatant is removed, five volumes of acetone–0.01 *M* HCl (60:40) are added and recentrifuged[14]. Supernatant is carefully removed and evaporated in a gentle stream of nitrogen. Residue is centrifuged at 3000 *g* for 5 min.

Porous polystyrene–divinylbenzene (PD) columns

Sample is dissolved in 4 ml of Tris–HCl buffer at pH 7.4 and placed on a PD column (0.5 g of Biobeads SM2 per g of tissue, Bio-Rad Labs, Richmond, CA, U.S.A., 20–50 μm mesh). PD column material is a 50:50 copolymer of polystyrene and divinylbenzene. Columns are 100 × 10 mm. A peptide fraction containing enkephalins is eluted with 6 ml of methanol in 2 ml fractions instead of a total of 0.9 ml as mentioned by other workers[15].

Octadecylsilica (ODS) mini-columns

ODS minicolumns are commercially available (Sep-Paks, Waters Assoc., Milford, MA, U.S.A.) prepackaged columns (0.4 g) used for reversed-phase (RP) chromatography[16]. An octadecyl group is chemically bound to the silica. The ODS minicolumn is prepared by washing with 4 ml of methanol, 4 ml of water, 4 ml of methanol, then 8 ml of trifluoroacetic acid (TFA, 0.5%)[17]. Sample is dissolved in 4 ml of 0.5% TFA and placed on the minicolumn. A peptide fraction is removed with 2 ml of acetonitrile–0.15% TFA (80:20).

Radioactivity counting

Radiolabeled enkephalin is determined by counting radioactivity (Hewlett-Packard Tri-Carb 460C) during several purification steps to determine recovery yield: protein precipitation, before and after P-D and ODS columns, and at the end of HPLC separation.

High-performance liquid chromatography

A Waters HPLC apparatus is used and consists of a U6K injector, guard column packed with Corasil B (30–50 μm diameter), two μBondapak C_{18} (10 μm diameter) reversed-phase analytical stainless-steel columns (30 cm × 4 mm I.D.) in series,

two Model 6000A solvent delivery pumps, a Model 600 solvent programmer, and a Model 450 variable-wavelength UV detector[8]. Analogue signals are recorded on a Houston potentiometric recorder.

A volatile HPLC buffer is used for resolution of peptides in biologic extracts. Dilute formic acid (0.04 *M*) is titrated to pH 3.15 with distilled triethylamine to form TEAF buffer[8]. Aqueous solutions are filtered and degassed with filters (0.45 μm pore diameter, HAWP 04700, Millipore, Bedford, MA, U.S.A.). Organic solutions are treated similarly (FHIP 04700, 0.5 μm pore diameter).

The peptide bond absorbance at 190–200 nm was monitored to optimize sensitivity of detection[9]. For example, HPLC of synthetic somatosatin solutions with TEAF at 190 nm yielded femtomole sensitivity[8].

Lyophilized biologic extracts are dissolved in TEAF (100 μl). After HPLC resolution into individual components, fractions are collected in non-silylated "reacti-vials" by switching the "waste–recycle–collect" valve to "collect". Collected volumes of solvent (several millilitres) are reduced with a gentle stream of nitrogen, then lyophilized. Use of non-silylated glassware is mandatory during this process to avoid shifts in mass values during FD-MS.

Field desorption mass spectrometry

A Finnigan (Varian) MAT 731 (Bremen, G.F.R.) mass spectrometer of Mattauch–Herzog double-focusing geometry outfitted with a field desorption–field ionization–electron ionization combination source is used. Static resolution is 1000; source temperature, 90°C; emitter potential, +8 kV; counterelectrode, −3 kV. Emitters are fabricated in our laboratory from 10 μm diameter tungsten wire by activation at high temperature in a benzonitrile atmosphere under influence of a high electric field. Carbon microneedle growth on the emitter wire surface extended to a length of *ca.* 30 μm. The instrument is focused on the FI spectrum of acetone.

Lyophilized HPLC fractions are dissolved in 100 μl of methanol to wash sides of the reacti-vial, and a gentle stream of nitrogen reduces sample volume to less than 10 μl. Sample is carefully transferred to the emitter with assistance from a microsyringe–micromanipulator–stereomicroscope ensemble[9].

FD emitters display no difficulty during desorption of synthetic peptide solutions. However, biologic matrix effects are manifested at this stage of analysis by a "stickiness" of the extract on the emitter. Due to the multi-step chromatographic procedures used, this "stickiness" may be due to the RP-ODS-HPLC column material, lipids, or other biologic matrix material. Care must be exercised during emitter heating to avoid mechanical breakage of the emitter.

The peak matching unit is set to scan alternately $(M+H)^+$ ions of LE at *m/z* 556 and ^{2}Ala-LE at 570. The emitter heating current is increased manually to 16 mA where enkephalins desorb optimally[9–12]. An oscillographic recording is obtained manually of the entire desorption envelope as peak switching continues. The entire process is monitored on an oscilloscope. Individual ion currents of the two $(M+H)^+$ ions are integrated manually by summing respective peak heights. Mass spectral ion signals are corrected for cross contributions due to isotopes and to fragmentation (contribution of 570 to 556 is 2% and 556 to 570 is 0.3%).

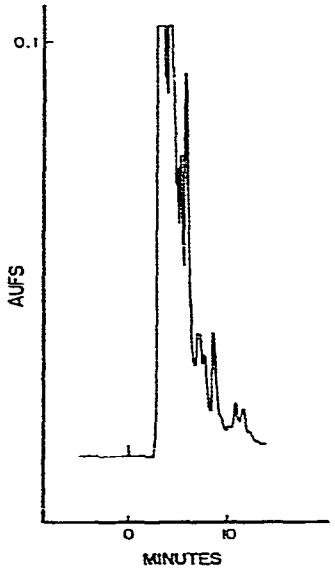
RESULTS

Fig. 1 contains the RP-HPLC chromatogram of unspiked canine thalamus tissue (2 g) containing internal standard. A second sample was spiked with 200 ng of LE per gram of tissue. Experimental chromatographic conditions are shown in the figure. The retention times for LE (7.1 min) and internal standard (10.6 min) are known and reproducible. A mixture of synthetic peptide standards was injected before biologic sample to accurately verify retention times.

Fig. 2 contains an outline of the scheme used to calculate endogenous amount of LE in brain tissue. A known amount of tissue is divided into two equal parts. One-half is subjected to Sep-Pak, Bio-Beads SM2, another Sep-Pak, HPLC, then FD-MS measurement. The other half is spiked with 200 ng of LE per gram of tissue and subjected to the same sequence of chromatography.

FD-MS measurement yields a ratio (R) of integrated $(M+H)^+$ ion currents due to LE and internal standard. The R_1 value is linearly proportional (slope = a) to endogenous amount of LE (x ng per g of tissue) in the unspiked sample. Preliminary data (not published) indicate that the intercept of this straight line equals zero. Whenever sample is spiked, the straight line shifts to the right 200 ng on the abscissa and the equation $a(x + 200) = R_2$ is used. The a and x values remain the same as in the unspiked analysis, while R_2 becomes the new ratio determined by FD-MS. Solution of the two equations with two unknowns yields an x value of ng LE per g of tissue. Use of spiked sample increases accuracy.

Data in Fig. 3 were obtained to determine the level of sensitivity achievable



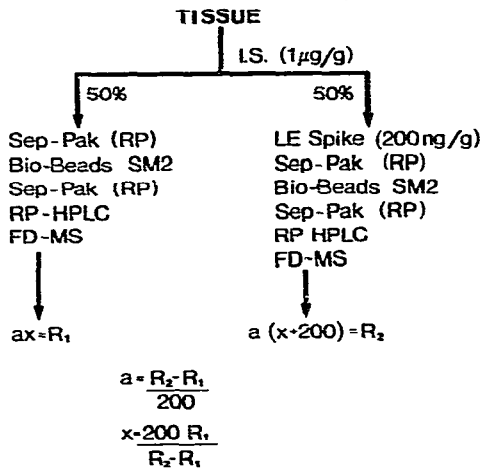


Fig. 1. RP-HPLC chromatogram of thalamus tissue extract. Two C_{18} columns; solvent, acetonitrile–TEAF (30:70), 1.5 ml/min; detection at 200 nm.

Fig. 2. Scheme used to calculate endogenous LE in spiked and unspiked thalamus tissue extracts.

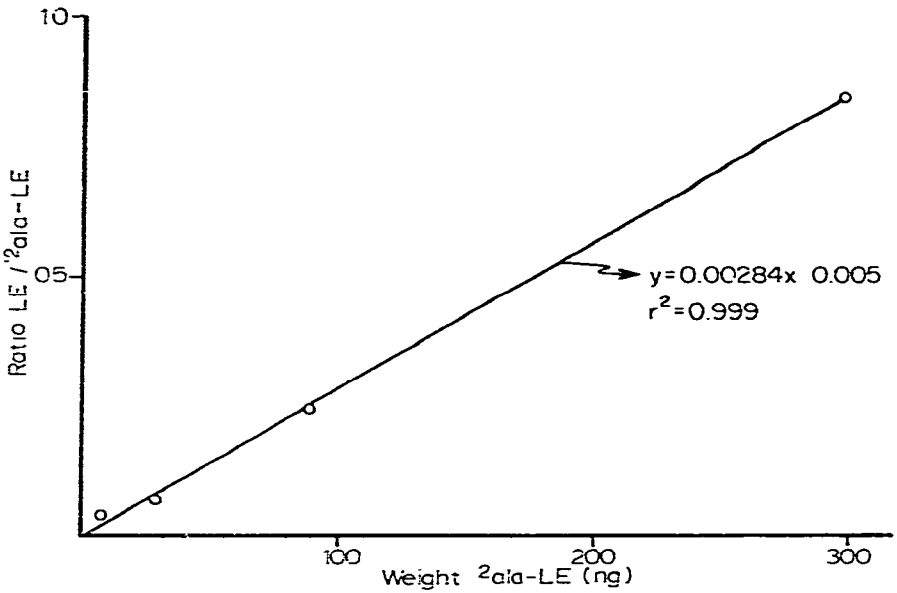


Fig. 3. Calibration curve of FD-MS measurement of synthetic LE/I.S. solutions.

with the combination of HPLC and FD-MS measurement of synthetic solutions of opioid peptides. Four samples were prepared where each sample contained 300 ng of internal standard, ^{2}Ala-LE. To each one of these four samples, successively lower amounts (300, 100, 30 and 10 ng) of LE were added to produce solutions with ratios of 1:1, 3:1, 10:1 and 30:1, LE to IS, respectively. FD-MS measurement of these four synthetic mixtures yielded data shown in Fig. 3. It can be clearly seen that down to 30 ng (60 pmol) LE can be measured. It must be remembered that all aspects of this measurement are done manually. Experience in our laboratory indicates that biologic matrix effects play a role in measurement of peptides in biological samples and the 30 ng level is not routinely obtained for biologic samples.

Field desorption (selected ion monitoring) measurement of LE in tissue extract

TABLE I

MEASUREMENT OF LE/IS ION CURRENT RATIOS (R) IN SPIKED AND UNSPIKED THALAMUS TISSUE EXTRACTS

	Sample				*Mean*
	1	*2*	*3*	*4*	
Unspiked	0.0528	0.0572	0.0587	–	0.0561
Spiked	0.1609	0.2785	0.3288	0.3202	0.2721

$ax = 0.0561$
$a(x + 200) = 0.2721$
$x = 51.92$ ng LE/g thalamus tissue

from canine thalamus was performed on HPLC fractions and data are collected in Table I. The data yield a value for amount of endogenous LE equal to 51.92 ng/g tissue or 52 ppb.

DISCUSSION

Leucine enkephalin is measured in canine thalamus tissue by a combination of instrumental analytical techniques in an effort to preserve molecular specificity of the measurement. HPLC efficiently and rapidly resolves Leu-enkephalin from a biologic tissue extract with high resolution. FD-MS of chemically underivatized LE efficiently provides a protonated molecular ion $(M+H)^+$ from picomole amounts of that neuropeptide and retains molecular structure information. Measurement of $(M+H)^+$ provides confidence that quantification of only that compound of interest is being performed. The sensitivity of this novel HPLC–FD-MS measurement is at the 50 ng LE/g tissue (50 ppb), or picomole amounts.

Other workers have measured brain peptides by RIA, and is some cases, preliminary chromatography was done. Variability in published methods of handling tissue and the time elapsed[18] following tissue procurement but before measurement are two highly variable and critical parameters that must be understood and controlled. Recent research indicates endogenous tissue proteinases, peptidases[19], enkephalinases[20], and synthetases are all present in tissue extracts. For that reason, rapid tissue procurement, temperature lowering, and protein precipitation to destroy all enzyme activity are important preliminaries to measure biologic peptides of interest to reflect accurately amounts of that specific peptide originally present in the biologic tissue.

Tissue is procured in a very rapid manner in our study and a specific method of handling that tissue was developed in this laboratory and applied to various brain tissues. Within minutes of neurosurgery, neuroanatomic identification of specific brain regions was followed by rapid excision of these individual brain regions and placement of tissue into liquid nitrogen followed by storage at $-70°C$. Enzyme activity of enkephalinases, proteinases, peptidases, and synthetases were minimized or completely abolished. Specific RP chromatographic techniques were utilized to take advantage of chromatographic hydrophobic properties of individual peptide:buffer molecular complexes. For example, for enkephalins, a polystyrene–divinylbenzene minicolumn[15] was utilized because of this column's propensity to retain enkephalins. Most other peptides are retained by ODS RP packing, and these minicolumns are utilized followed by ODS RP-HPLC columns.

Biologic matrix effects observed with brain and tooth-pulp tissue demand that an internal standard be used for each neuropeptide quantified. Higher homologs have been used with success in this study. Stable isotope-labeled internal standards are also possible.

Recovery studies were performed with tritiated peptide (LE) tracers to determine accurately recovery of individual peptides. Close scrutiny of the Materials and Methods sections in many published papers failed to reflect accurately what is the overall recovery of a peptide from biologic tissue. Many workers do not equilibrate IS with tissue overnight[13]. We equilibrate tracer and IS overnight to ensure optimal penetration into biologic matrix and mixing of endogenous peptide with exogenous tracer.

The most important parameter obtained by methods outlined here include the molecular parameter demanded for any accurate measurement of an endogenous biologically active peptide or metabolite in biologic tissue or fluid. FD-MS produces a protonated molecular ion $(M+H)^+$ which relates to the total molecular information of the molecule being quantified. Fast atom bombardment is another ionization method[21]. For further increased molecular specificity, other instrumental techniques (collision activation, linked scanning, and high-resolution MS) can be utilized[22]. A microprocessor system is being fabricated to control emitter heating current and ion current integration. Microprocessor-controlled data acquisition will reduce errors and increase sensitivity.

Analytic measurements of LE in thalamus tissue described here are compatible with our previously published RP-HPLC–FD-MS measurements of LE and Met-enkephalin in brain tissue (caudate nucleus and hypothalamus[10]) and tooth pulp[11]. Current measurements extend our experience to achieve our goal to quantify several neuropeptides in several brain regions. Furthermore, the results from RP-HPLC indicate that other peptides remain to be sequenced and quantified. Sequencing is required in an effort to (a) determine all bioactive peptides in these tissues, (b) provide a "metabolic profile" to describe normal and pathological conditions, and (c) describe the biosynthetic precursors and metabolic products of active neuropeptides. Having been sequenced, individual peptides can then be quantified.

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LIST OF ABBREVIATIONS

B:E	link scanning
CA	collision activation
CI	chemical ionization
EI	electron ionization
FAB	fast atom bombardment
FD-MS	field desorption-mass spectrometry
fmol	femtomole (10^{-15} mole)
HPLC	high-performance (pressure) liquid chromatography
LE	leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu)
mA	milliampere
m/z	mass-to-charge ratio of an ion
$(M+H)^+$	protonated molecular ion
ODS	octadecylsilica
ppb	parts per billion
PD	polystyrene–divinylbenzene
pmol	picomole
RIA	radioimmunoassay
RP	reversed-phase
TEAF	triethylamine–formic acid
TFA	trifluoroacetic acid

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