Journal of Chromatography, 342 (1984) 245–260
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 342

MEASUREMENT OF ENKEPHALIN PEPTIDES IN CANINE BRAIN REGIONS, TEETH, AND CEREBROSPINAL FLUID WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

DOMINIC M. DESIDERIO*

Department of Neurology and the Charles B. Stout Neuroscience Mass Spectrometry Laboratory, College of Medicine, University of Tennessee Center for the Health Sciences, 800 Madison Avenue, Memphis, TN 38163 (U.S.A.)

MASAAKI KAI

The Charles B. Stout Neuroscience Mass Spectrometry Laboratory, College of Medicine, University of Tennessee Center for the Health Sciences, 800 Madison Avenue, Memphis, TN 38163 (U.S.A.) and Department of Chemistry, Kyushu University, Fukuoka (Japan)

FRANCIS S. TANZER

Department of Biologic and Diagnostic Sciences, College of Dentistry, University of Tennessee Center for the Health Sciences, 800 Madison Avenue, Memphis, TN 38163 (U.S.A.)

and

JOANN TRIMBLE and CLAIRE WAKELYN

The Charles B. Stout Neuroscience Mass Spectrometry Laboratory, College of Medicine, University of Tennessee Center for the Health Sciences, 800 Madison Avenue, Memphis, TN 38163 (U.S.A.)

SUMMARY

Endogenous enkephalin pentapeptides are measured with unambiguous molecular specificity in canine and human tissue and fluid extracts. Both field desorption and fast atom bombardment mass spectrometry have been used to produce a protonated molecular ion of the peptide high-performance liquid chromatography fraction. The protonated molecular ion is subjected to collision-activated dissociation processes and a linked-field scan (B/E) selects a unique amino acid sequence-determining ion for monitoring and measurement. Stable isotope-incorporated peptide internal standards are used for quantification. Endogenous enkephalins are measured in hypothalamus, cerebrospinal fluid, pituitary, caudate nucleus, and tooth pulp extracts. Part-per-billion levels of endogenous peptide are measured.

INTRODUCTION

The objective of this manuscript is to describe an analytical procedure for measurement in biological tissues and fluids of the two endogenous opioid pentapeptides leucine enkephalin (LE = YGGFL)* and methionine enkephalin (ME = YGGFM) with maximum molecular specificity attached to that measurement. Quan-

* Abbreviations: Y = tyrosine, G = glycine, F = phenylalanine, L = leucine, M = methionine.

0021-9673/84/$03.00

tification of these two opioid peptides is a component of a larger study aimed towards accurately describing the molecular events occurring in pain processes. In an effort to unambiguously characterize those molecular events, analytical data are presented on the endogenous amounts of those two pentapeptides in five tissues (canine tooth pulp, hypothalamus, pituitary, caudate nucleus, and tooth pulp) plus cerebrospinal fluid (CSF). To facilitate the measurement process in a fast and facile manner, the mass spectrometer is interfaced to a microcomputer to acquire data objectively.

The first hypothalamic neuropeptide sequenced was the thyrotropic releasing factor, TRF[1]. Since then, the amino acid sequences of other neuropeptides have been determined, including somatostatin[2], beta-endorphin, dynorphin[3], luteinizing releasing factor[4], cholecystokinin octapeptide, amongst others. An endogenous ligand for the morphine receptor was discovered and found to be comprised of two opioid pentapeptides—leucine enkephalin and methionine enkephalin[5]. Since then, it has been found that three peptidergic pathways (endorphinergic, enkephalinergic, and dynorphinergic) are available to an organism for its attempts to deal with noxious stimuli[6].

Each one of these peptidergic pathways is comprised of a family of closely-related peptides, including large precursors[7–10], intermediate precursors, the biologically active pentapeptides, and inactive metabolites. Several enzymes operate in these peptide pathways and for enkephalins, for example, include enkephalinase[11,12] carboxypeptidase[13], and aminopeptidase[14,15]. One hypothesis states that opiod peptides play a neuromodulatory role where the firing rate for substance P neurons is decreased by the enkephalins.

Because of the important role that brain peptides in general and enkephalins in particular play in both brain neurochemistry and pain mechanisms, it is imperative to measure endogenous peptides with unambiguous molecular specificity[16]. A variety of analytical methods is used in most laboratories to attempt to achieve that goal of high molecular specificity. However, that goal is rarely, if ever, met. It is important to consider the fact that if a chromatographic procedure has infinitely high resolution, no specific detector is required because the analyst could depend on a unique retention time for structural assignment. On the other hand, if the molecular specificity of the detector were infinitely high, a chromatographic resolution step would not be required because the structure of the molecule would be determined in the detection process itself. While molecular specificity in the real world of analysis of endogenous compounds in a biological matrix is less than infinite, it is interesting to note that recent developments in mass spectrometry are tending towards the latter situation where tandem mass spectrometry (mass spectrometry mass spectromety, MS–MS) offers optimum structure information[17].

Several methods of peptide measurement are available. Reversed-phase high-performance liquid chromatography (HPLC) is commonly used with a variety of detectors, including fluorescent, UV, electrochemical, and refractive index detectors. It must be remembered that unambiguous molecular structure can not be measured by, or inferred from, the output from these types of chromatographic detector. These detectors can only respond to a secondary structural characteristic of the molecule, such as the presence of a chromophore or an electroactive functional group. A second analytical method for peptide measurement is bioassay, such as the assays which employ the guinea pig ileum or mouse vas deferens. Again, the molecular specificity

of this procedure is quite low, even though sensitivity is high[18]. A third assay, radioreceptor assay, is an elegant method used to discover the endogenous ligands for the morphine receptor and is also used as a low molecular specificity–high sensitivity screening method for a variety of chromatographically-purified components from biologic tissue extracts and fluids[19–21]. A fourth assay method, radioimmunoassay (RIA), provides one of the highest sensitivity levels and a higher molecular specificity detection system compared to the other methods in the above list. However, RIA is limited by the fact that a highly specific antibody must be raised to each and every ligand to be measured in a biological matrix, and even then, specificity and avoidance of cross-reactivity to specific or non-specific compounds cannot be rigorously guaranteed. While some workers combine HPLC with RIA measurement[22], it still must be remembered that molecular specificity is not optimal[23].

Several workers utilize an off-line combination of high-performance chromatographic separation and analysis by several detection systems which include RIA[22,23] radioreceptor assay[21], and mass spectrometry[24–30].

Mass spectrometry is the method of choice for analysis whenever maximum molecular specificity of the measurement process is desired and whenever sufficient analyte is available[24–31]. Even when mass spectrometry is not used for routine analysis, at least an analytical standard is now available to calibrate all of the other assay methods. While electron ionization has been utilized for analysis of derivatized pentapeptides, newer ionization techniques make mass spectrometry quite amenable to measurement of underivatized endogenous peptides. Field desorption mass spectrometry readily produces a protonated molecular ion which, in conjunction with a stable isotope-incorporated internal standard, is utilized for peptide measurement[24]. On the other hand, fast atom bombardment (FAB) mass spectrometry[32] is a gentle ionization process used to produce an abundant protonated molecular ion current which, in conjunction with collision-activated dissociation (CAD) and linked field (B/E) scan methodology[26], produces an amino acid sequence-determining ion generated specifically from only the protonated molecular ion of the target peptide. The amino acid sequence-determining ion is measured in the selected ion monitoring (SIM) mode. The highest level of molecular specificity obtained for measurement of endogenous peptides therefore, is the novel combination instrumental system FAB–CAD–B/E–B'/E'–SIM–microcomputer analysis of a specific amino acid sequence-determining ion[26].

While it is granted that mass spectrometric techniques are costly (only the initial capital investment and not subsequent unit measurement cost nor rapid amortization), technically complicated, and demand a high level of technical expertise compared to the other assays discussed above, nonetheless, mass spectrometry does offer for the first time a needed independent calibration method for the other assay methods.

MATERIALS AND METHODS

Animal model

The mongrel dog (six months to one year old; average weight, 13.5 kg) is the animal model and a small number of animals (up to three) is used for these studies. On the other hand, for the pituitary measurement, 27 and 40 pituitaries have been accumulated for the two studies discussed below, respectively.

Tissue procurement

The dog is treated with pentobarbital before exsanguination. The dog is exsanguinated via a femoral artery to decrease the amount of blood remaining in the various tissues during necropsy. The brain is removed from the cranium and individual brain regions are neuroanatomically identified and excised. The pituitary and teeth are rapidly removed and a hypodermic needle is used to remove CSF from the top of the spinal column. All brain tissues and CSF are frozen rapidly in liquid nitrogen to avoid any metabolic or chemical interconversion. Tooth pulp tissue is homogenized immediately upon removal.

Tooth pulp electrostimulation

A standard dental clinic tooth pulp tester is used for electrostimulation. The tooth pulp tester is applied to the tooth at the incisal edge for 2 sec after cannulation but before exsanguination.

Internal standard

To minimize experimental differences observed in both the hydrophobicity during reversed-phase HPLC separation and in the mass separation between the protonated molecular ions of the peptide of interest and internal standard, a stable isotope-incorporated enkephalin internal standard is utilized[24–33]. The $^{18}O_2$ species is added (200 ng per gram of tissue or millilitre of fluid). In contradistinction to the advantages noted above of the stable isotope-incorporated peptide internal standard, one disadvantage is that the acidic conditions previously developed for use during homogenization must now be avoided and neutral conditions (methanol) are used to avoid back-exchange of ^{18}O incorporated into the peptide internal standard for endogenous ^{16}O atoms from aqueous solutions[24].

Chromatography

Proteins are precipitated with methanol and removed by centrifugation. Supernatant is applied to a reversed-phase Sep-Pak cartridge (Waters, Milford, MA, U.S.A.). While it might be thought that a peptide does not possess sufficient hydrophobicity to efficiently participate in reversed-phase separation processes, appropriate ion-pairing techniques ensure that the ion-pair–peptide complex is preferentially retained on the C_{18} hydrophobic surface of both the Sep-Pak and the analytic column[27]. The Sep-Pak surface is first prepared by washing (4 ml of methanol, 4 ml of water, 4 ml of methanol), a Tris-wash follows, the supernatant is applied in a Tris buffer (50 m*M*, pH 7.5), and peptides eluted by stepwise increases of ethanol. The last (fourth) fraction is eluted with Tris buffer–ethanol (20:80). The eluted peptide-rich fraction is injected onto an analytic steel reversed-phase HPLC column. The isocratic HPLC apparatus (Waters) consists of a U6K injector, M6000 pump, reversed-phase C_{18} analytic steel column, and UV detector (Model 450). Fractions are collected manually at the retention times noted for the two synthetic peptides YGGFM and YGGFL, respectively. The aqueous buffer used in this study is volatile, a requirement of the subsequent analytical methods, and is comprised of 0.04 *M* formic acid titrated to pH 3.2 with distilled triethylamine[34]. The organic modifier is acetonitrile.

Each fraction collected is divided into two parts. One part is subjected to a

sequence of Sep-Pak, analytic HPLC, and mass spectrometric measurement while the second part is subjected to the same sequence, but after being spiked with a known amount of [^{16}O]enkephalin. The amount of endogenous peptide (x) is calculated from the equation $x = y \cdot R_1/(R_2 - R_1)$, where y = amount of [^{16}O]enkephalin spike (ng per tissue) and R_1 and R_2 are the two measured ratios of protonated molecular ion currents (endogenous and internal standard) for both unspiked and spiked samples, respectively.

Mass spectrometry

A Finnigan MAT double-focusing,forward (E,B) geometry mass spectrometer is utilized. Nominal resolution is 1000 for both field desorption and FAB mass spectrometry measurements. The ion source remains at ambient temperature. The peak-matching unit of the mass spectrometer is utilized to alternately measure the $(M+H)^+$ of the endogenous peptide and internal standard. The timing sequence of accelerating voltage alternation is *ca.* 2 sec cycle^{-1} over the duration of the ionization of amount of the peptide.

For field desorption mass spectrometry, emitters are prepared using benzonitrile, and sample is deposited onto the emitter with a micromanipulator–microscope–microsyringe ensemble. The protonated molecular ion of the peptide of interest is monitored in alternation with the $(M+H)^+$ of the $^{18}O_2$ internal standard located four mass units higher. The peak-matching unit alternates between these two $(M+H)^+$ masses and the on-line microcomputer[35] accumulates the ion current measured at each individual mass. In contradistinction to the FAB mass spectrometric data discussed below, the signal-to-noise ratio obtained with field desorption permits direct comparison of the two $(M+H)^+$ signals. Many (50–70) scans can now be acquired objectively by the mass spectrometer–microcomputer interface to provide a fast and facile method to reduce operator error and fatigue and to improve the accuracy of the measurement[35].

FAB mass spectrometry

The principles of FAB mass spectrometry are outlined elsewhere[32], a detailed description of the conversion of a Finnigan MAT 731 mass spectrometer to the FAB ionization mode is described[36], and the utilization of FAB for peptide analysis is described[37]. Xenon is utilized as the bombarding particle, because the kinetic energy due to its large atomic weight produces the greatest intensity of protonated molecular ion current relative to fragmentation compared to argon. The kinetic energy of the xenon fast atoms is 8.5 keV and the current is *ca.* 50 μA. Glycerol is utilized as the non-volatile matrix in which the peptide fraction is dissolved[38]. Because glycerol produces its own mass spectrum, which is superimposed on the mass spectrum of the peptide, it is necessary to extract peptide amino acid sequence-determining information from the FAB-produced mass spectrum by means of combined collision activated dissociation-linked field (B/E) scanning methods[27]. While high resolution data could be used to provide accurate mass measurement but not structure data for $(M+H)^+$, collision-activated dissociation produces and linked field (B/E) scanning selects a specific amino acid sequence-determining ion. This MS–MS process[17] virtually eliminates any potential interferences and ambiguities in the measurement of endogenous peptides[26]. For example, the HPLC fraction eluted at the retention time

corresponding to that for a synthetic peptide standard (*e.g.*, YGGFL) is collected. After buffer and organic modifier are lyophilized, that fraction is placed on the FAB probe tip in a glycerol matrix. Fast xenon atoms produce an abundant $(M+H)^+$ ion current at nominal mass 556 for leucine enkephalin. The $(M+H)^+$ ion, following acceleration to 8 kV, is subjected to collision-activated dissociation processes where the ion kinetic energy is transferred to higher electronic levels[39], to induce bond fragmentation[37]. A specific amino acid sequence-determining ion at mass 336, representing the C-terminal tripeptide fragment -GFL from YGGFL, is selected for monitoring. That ion shifts cleanly to mass 340 in the spectrum of $^{18}O_2$-leucine enkephalin internal standard. These two selected and specific amino acid sequence-determing ions, 336 and 340, are alternately collected, the ratio of the two accumulated ion currents is calculated, and the amount of $^{18}O_2$ internal standard is used to multiply the ratio and calculate the amount of endogenous enkephalin.

In the FAB–CAD–*B*/*E*–*B*′/E′–SIM–microcomputer mode, the calibration curve shown in Fig. 1 demonstrates linearity over the concentration range of analytical interest. The sensitivity of both the field desorption $(M+H)^+$–SIM and the FAB–CAD–*B*/*E*–*B*′/E′–SIM–microcomputer mode are comparable and can be used down to 30 ppb or 30 ng of peptide per gram of tissue. Current instrumental developments indicate a sensitivity to the pg g^{-1} level.

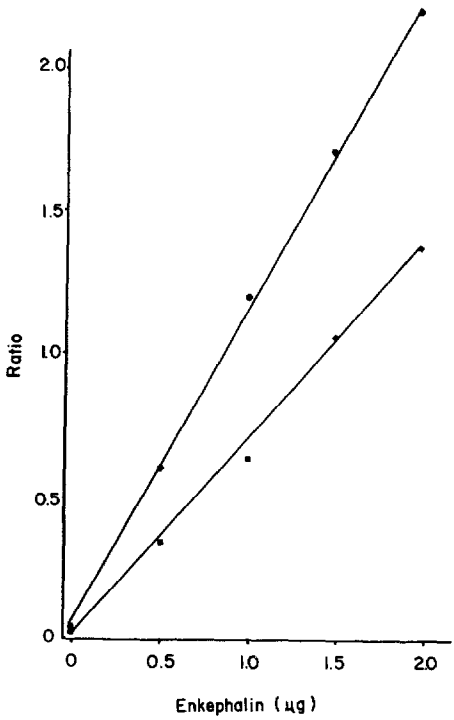

Fig. 1. Best-fit straight line calibration ion curves for leucine enkephalin (lower) and methionine enkephalin (upper), produced from integrated M_S/M_L ratios derived from FAB–CAD–*B*/*E*–*B*′/E′–SIM measurements on 336 (LE) and 378 (ME). For LE, $y = 0.69x$ and $r^2 = 0.995$; for ME, $y = 1.09x + 0.06$ and $r^2 = 0.999$.

Microcomputer

An Apple II-plus microcomputer is electronically interfaced to five sections of the mass spectrometer: the oscilloscope, accelerating voltage alternator, total ion current monitor, emitter heater current, and the electron multiplier output[35]. The *B*/*E*–*B*′/E′ alternating mode is effected by changing the two respective fields in an appropriate manner. The number of scans generally ranges between 50 and 70, a number sufficient to define good statistics of the measurement compared to previous manual measurement methods involving limited numbers[29,30]. The microcomputer minimizes operator fatigue and error and makes the entire measurement process fast (only minutes to obtain the data), facile (no analog data is acquired or measured), and objective (computer software defines areas, thresholds, and calibration).

RESULTS AND DISCUSSION

Calibration

The method of analysis employing the FAB–CAD–*B*/*E*–*B*′/E′–SIM–microcomputer demonstrates a linear response over the range of concentrations of endogenous peptides found in biological extracts. The use of both the stable isotope-incorporated internal standard and this novel analytical measurement mode is important to demonstrate linearity and to overcome biological matrix effects. Primary data from one of the accelerating voltage alternation experiments is shown (Fig. 2) for leucine enkephalin (2 μg) *versus* O^{18} leucine enkephalin (2 μg)[35]. The monitored peak is mass 336 in the former case and 340 in the latter case. This selected ion corresponds to the tripeptide sequence –GFL, which derives from the collision-activated dissociation/linked field (*B*/*E*) scan mode. This amino acid sequence-determing ion is a product ion, arising only from the protonated molecular ion (556) of leucine enkephalin. In the oscillographic trace (Fig. 2), the ^{18}O species is clearly indicated when the higher mass (M_S) is being monitored, because a 60-Hz signal is superimposed. The microcomputer accepts the data for the large number of signals to optimize ion statistics. Integrated areas are plotted, and the ratio of the known amounts of the ^{16}O and the ^{18}O species is plotted. Fig. 1 contains the calibration curves[35] for both methionine enkephalin (upper) and leucine enkephalin (lower). Both curves intercept at, or very near, the origin and have correlation coefficients near unity. The statistical parameters

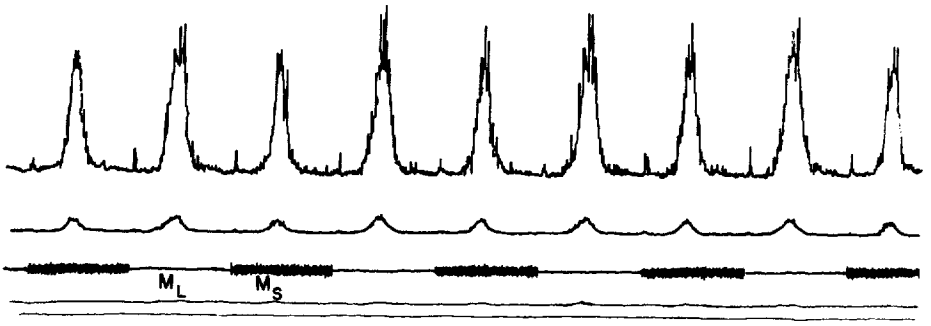

Fig. 2. Analog data for leucine enkephalin derived from the FAB–CAD–*B*/*E*–*B*′/E′–SIM–microcomputer mode. The peaks, which are alternately monitored, are due to the C-terminal fragment –GFL at M_L = 336 (LE) and M_S = 340 ($^{18}O_2$–LE).

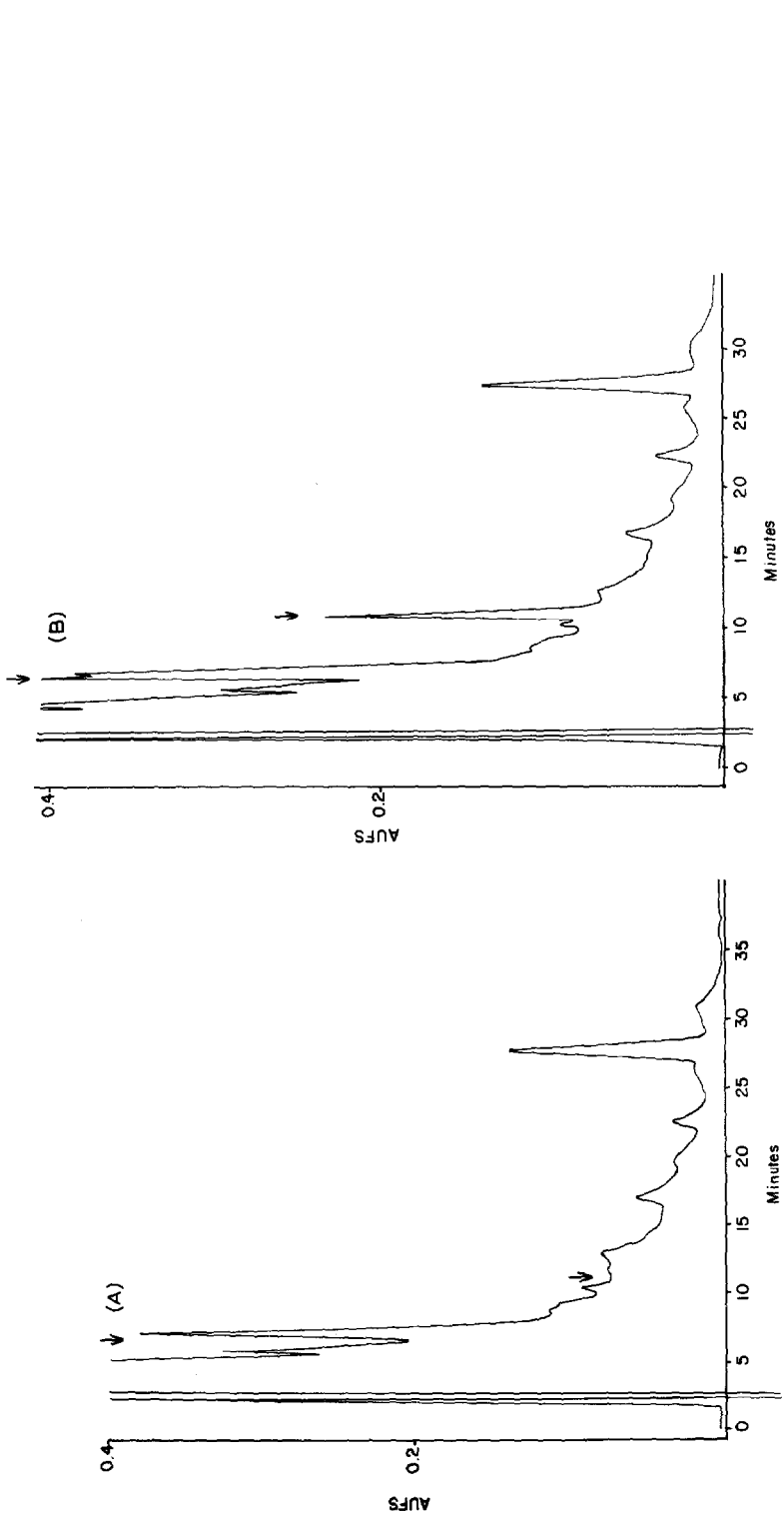

Fig. 3(A). Reversed-phase HPLC chromatogram of 2 g of canine hypothalamus tissue extract. Arrows indicate retention times for ME and LE, respectively. (B) Reversed-phase HPLC chromatogram of 2 g of canine hypothalamus tissue, extract spiked with $^{18}O_2$–ME and $^{18}O_2$–LE.

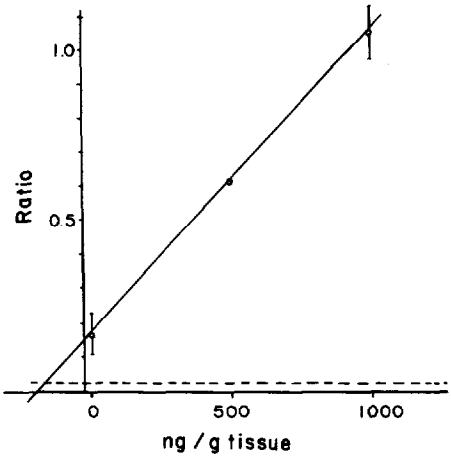

Fig. 4. Plot of FAB–CAD–*B*/*E*–*B*′/E′ SIM data for LE and $^{18}O_2$–LE in hypothalamus extract.

for the two best-fit straight lines are: for ME: $y = 1.09x + 0.06$, $r^2 = 0.999$; and for LE: $y = 0.69x$, $r^2 = 0.995$.

Hypothalamus

The reversed-phase high-performance liquid chromatogram for canine hypothalamus tissue (2 g) is shown in Fig. 3A and the chromatogram of sample spiked with the two enkephalin peptide internal standards $^{18}O_2$–ME and $^{18}O_2$–LE is shown in Fig. 3B. The two arrows indicate the known retention times of synthetic ME and LE, respectively. It should be noted that at the UV wavelength being monitored (200

TABLE I

MEASUREMENT OF ENKEPHALINS IN CANINE TISSUE EXTRACTS (ng g^{-1}) AND CSF (ng ml^{-1})

Source	*Enkephalin*	*Amount*	*Method**
Hypothalamus	LE	170	1
Cerebrospinal fluid	LE	44	1
Pituitary			
Anterior	LE	70	2
	ME	2950	2
Posterior	LE	2	2
	ME	3760	2
Caudate nucleus	LE	1500	2
Tooth pulp**	LE	30	2
	ME	179	2
Tooth pulp			
Control	LE	20	2
	ME	487	2
Electrostimulated	LE	45	2
	ME	390	2

* Method 1:FDMS–SIM–microcomputer; Method 2: FAB–CAD–*B*/*E*–*B*′/E′–SIM–microcomputer.
** Unstimulated pooled tissue from five dogs.

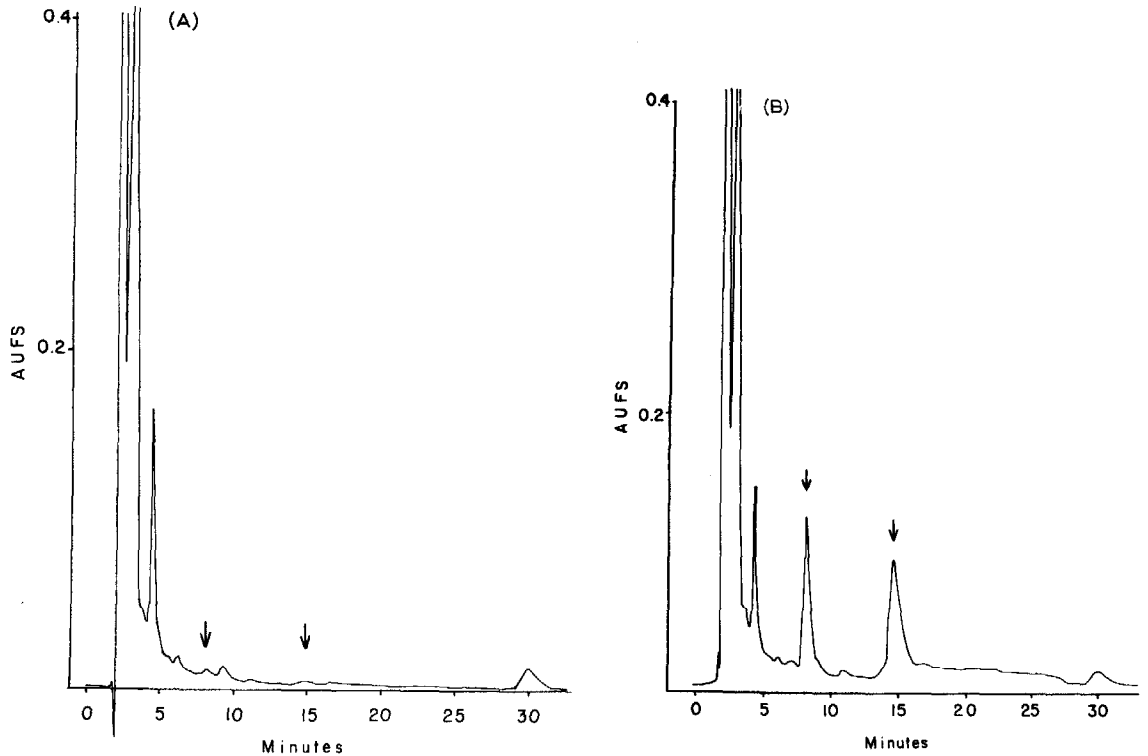

Fig. 5 (A). Reversed-phase HPLC chromatogram of 2 ml of canine CSF. (B) Reversed-phase HPLC chromatogram of 2 ml of canine CSF, spiked with $^{18}O_2$–ME and $^{18}O_2$ LE.

nm), the reversed-phase HPLC chromatogram of a hypothalamic peptide-rich fraction is relatively clean.

While experiments indicate that the reversed-phase HPLC resolution of the two enkephalin peaks may be increased (recycling, buffer and/or organic modifier change, flow-rate, temperature, etc.), it must be remembered that in our mode of analysis, the detector is not limited to only UV absorption, but rather is based on the measurement of a unique amino acid sequence-determining ion arising from a peptide eluted at one selected retention time. In this type of measurement mode, virtually all chromatographic and chemical background noise disappears. The plot of leucine enkephalin and the ^{18}O leucine enkephalin internal standard $(M+H)^+$ ratios for this hypothalamus data is shown in Fig. 4, where intersection occurs at the noise level (dotted line) and corresponds to 170 ng of leucine enkephalin per gram of hypothalamus tissue. Table I summarizes these measurements, and all of the analytical data which are discussed below in greater detail.

Cerebrospinal fluid

Fig. 5A shows the reversed-phase high-performance liquid chromatogram of canine CSF alone (2 ml) and Fig. 5B the chromatogram of CSF spiked with the two peptide internal standards. Both leucine enkephalin (44 ng ml^{-1}) and methionine enkephalin (84 ng ml^{-1}) are measured in this extract.

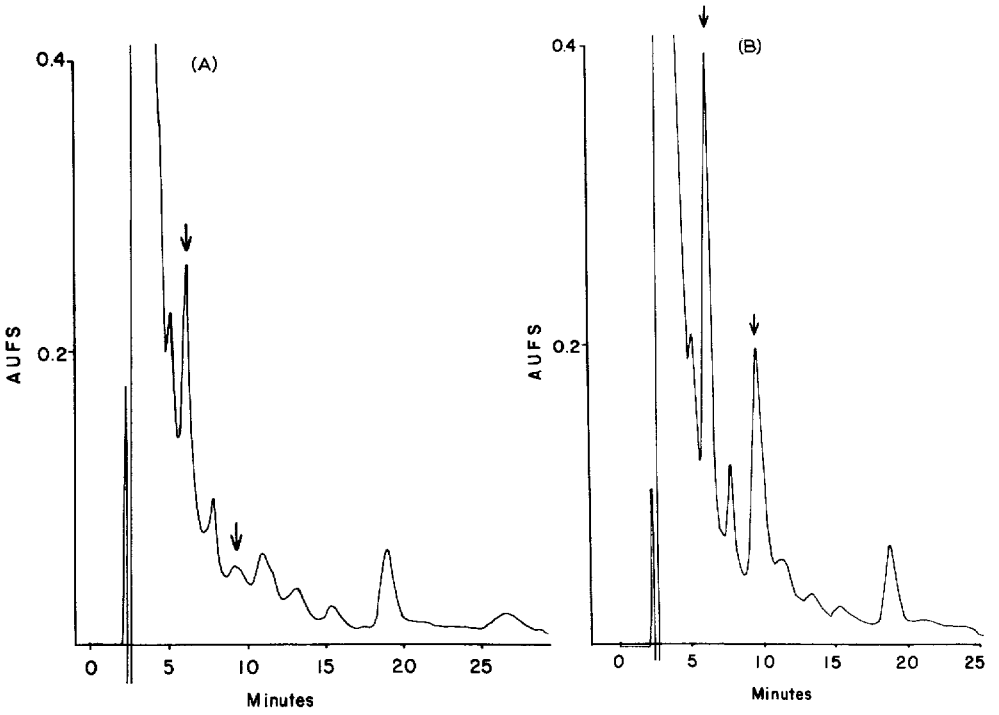

Fig. 6 (A). Reversed-phase HPLC chromatogram of 1.9 g of anterior pituitary tissue extract. (B) Reversed-phase HPLC chromatogram of 1.9 g of anterior pituitary tissue extract, spiked with $^{18}O_2$-ME and $^{18}O_2$-LE.

Pituitary

A number (40) of canine pituitaries were accumulated and neuroanatomically separated into the anterior (1.9 g total wet weight) and posterior (0.44 g total wet weight) portions. The tissue was homogenized in acetic acid (1 *M*) and divided into three equal samples. Fig. 6A shows the reversed-phase high-performance liquid chromatogram of the anterior pituitary tissue extract alone and Fig. 6B the anterior pituitary extract plus two peptide internal standards. Figs. 7A and 7B show the corresponding reversed-phase HPLC chromatograms for the posterior pituitary extraction. Table I lists the amounts of the enkephalins found in both the anterior (LE = 70; ME = 2950 ng g^{-1}) and in the posterior (LE = 2; ME = 3760 ng g^{-1}) pituitary extracts.

Caudate nucleus

This laboratory has reported previously the endogenous amount of enkephalin measured in canine caudate nucleus tissue extracts determined by means of field desorption mass spectrometry methods[29,30]. The amount of endogenous enkephalin in the canine caudate nucleus tissue extract in the current analytical study was 190 of methionine enkephalin and 1500 ng of leucine enkephalin per gram of tissue.

Tooth pulp

Pooled tooth pulp tissue was collected from four animals (four teeth in each animal, total = 16). Canine tooth pulp reversed-phase HPLC chromatograms have

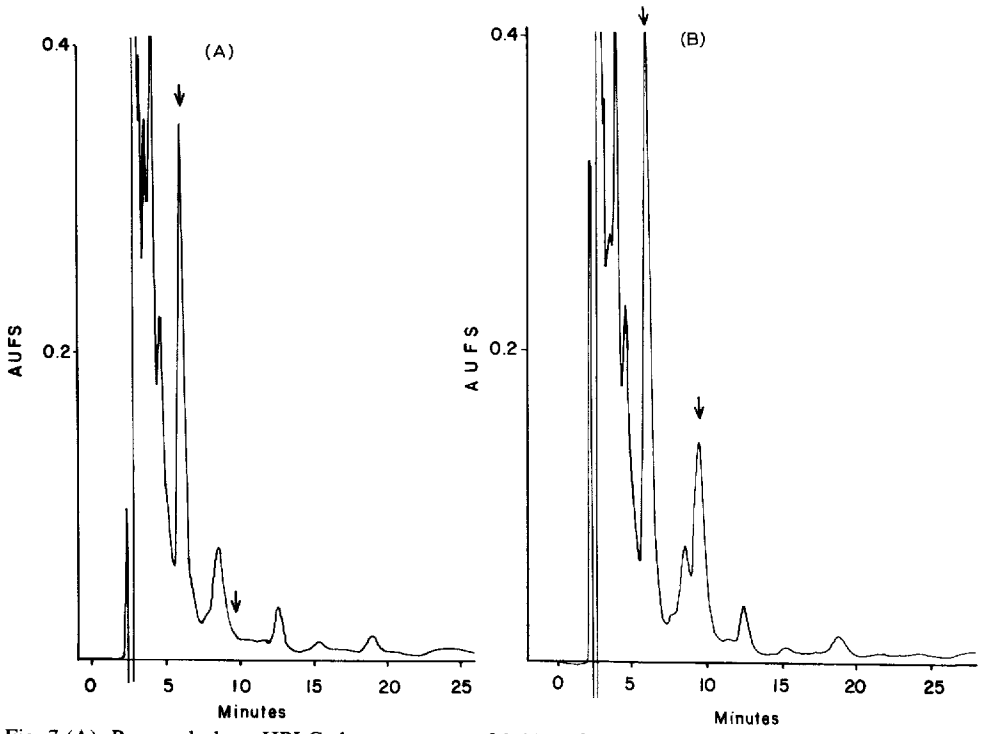

Fig. 7 (A). Reversed-phase HPLC chromatogram of 0.44 g of posterior pituitary tissue extract. (B) Reversed-phase HPLC chromatogram of 0.44 g of posterior pituitary tissue extract, spiked with $^{18}O_2$–ME and $^{18}O_2$–LE.

TABLE II

INDIVIDUAL MASS SPECTROMETRIC MEASUREMENTS OF ENKEPHALINS IN CANINE TOOTH PULP EXTRACTS

Data in nanograms of enkephalin per gram of tissue.

	Dog	*ME*	*LE*	*Wet weight (g)*
Control, unstimulated	112	750	30	1.714
	114	90	9	2.090
	127	620	20	1.071
Stimulated	110*	690	120	0.528
	115	390	10	1.637
	134	90	6	1.711

* Exsanguination begun before electrostimulation.

been published[28]. The endogenous amount of enkephalin for pooled tooth pulp tissue was 179 ng of methionine enkephalin and 30 ng of leucine enkephalin per gram of tooth pulp tissue.

Electrostimulated tooth pulp

In a study to determine whether electrical stimulation affects enkephalin concentration, three animals served as controls and three for electrostimulation experiments. Table II collects the individual analytical measurements for this study. While it is noted that animal 110 was exsanguinated before stimulation and relatively less tissue (0.528 g) was obtained, we have no basis for rejecting these data at this time. Also, ME data for animals 112 and 110 seem to be proportionately higher due to perhaps a lower level of internal standard in the mass spectrometric measurement, even though the HPLC data appear acceptable. These two questions notwithstanding, it is noted (Table I) that electrostimulation significantly decreases by 20% the amount of endogenous methionine enkephalin while doubling the amount of leucine enkephalin.

The general trend derived from these analytical data is that both of the opioid pentapeptides methionine enkephalin and leucine enkephalin are altered upon electrostimulation[40,41]. Electrostimulation is performed in an effort to elucidate the molecular mechanisms operating during a physiologically stressful situation. This electrostimulation experiment may or may not be exactly the same molecular process that occurs during endogenous pain. Nonetheless, our data indicate that the three peptidergic pathways (dynorphinergic, endorphinergic, enkephalingeric) may be mobilized in the following sequence: Large precursor → intermediate precursor(s) → pentapeptides(s) → inactive metabolites[6]. On one hand, there may be a naturally-occurring pool of pentapeptides which is electrostimulated towards metabolism or, on the other hand, the entire metabolic scheme noted above may be stimulated, leading to a lowered endogenous amount of each constituent peptide. Other human[23] and *in vivo* dynamic studies are needed to resolve that question.

CONCLUSIONS

Several conclusions are derived from the experiments reported in this manuscript.

A fast and facile method of sample acquisition and procurement from the canine animal model is described. This study demonstrates the need for rapid freezing of tissues in a fashion similar to that described for the discovery of 19OH-PGE_2 compounds in human seminal fluid[42]. Rapid freezing avoids, or at least minimizes, both metabolic and chemical interconversions and also enhances the possibility of measuring only those endogenous target compounds but not artifacts nor chemical/enzymic products.

The need for an internal standard for mass spectrometric measurement has been demonstrated by other workers using mass spectrometric measurement[43] to overcome biological matrix effects. Stable isotope-incorporated compounds are the internal standards of choice, because they represent the molecular structure closest to the endogenous peptides and the also have similar hydrophobicity and mass spectrometric behavioral characteristics. An internal standard is added as soon as possible after tissue acquisition and before homogenization in the separation scheme to accurately represent the endogenous amount of peptide and also to provide sufficient time for equilibration[44] of the exogenous and endogenous peptides.

The most significant parameter of any analytical measurement of any biological compound is the molecular specificity of that measurement; namely, is the compound one thinks is being measured the compound that actually is being measured? This concept is easy to state but experimentally rather difficult to unambiguously prove. Many other assay methods are generally utilized because of their relative ease, low cost, high speed, high sensitivity, and putatively, high molecular specificity. For example, chromatography, color reactions, enzymatic reactions, HPLC, bioassay, radioreceptor assay, and RIA are all measurement procedures which are used in most laboratories around the world. We state unequivocally that the molecular specificity of all of the above measurement procedures is insufficient for unambiguous structural proof. Only one measurement process, namely mass spectrometry, offers unambiguous molecular specificity.

The sensitivity of the novel mass spectrometric process described above is quite sufficient for analytical measurement of endogenous peptides in most biological tissues and fluids. For example, enkephalin peptides in canine caudate nucleus tissue extracts are measured at the 200–400 ppb level, where the instrument may limit measurement to the 30 ppb level. It is significant to realize that several significant instrumental improvements are being made and that an improvement in sensitivity of a factor of perhaps up to 100 may be expected soon.

The peptide measurements tabulated in Table I include endogenous amounts of both methionine enkephalin and leucine enkephalin pentapeptides in hypothalamus, CSF, anterior and posterior pituitary, caudate nucleus, and tooth pulp (pooled and electrostimulated). This type of analytical/physiological study is undertaken in one of our laboratories to ensure quality control over all experimental manipulations, ranging from the live animal model through exsanguination, tissue procurement, homogenization, chromatography, mass spectrometry, and data analysis. Inter-animal biological variations are observed and it is possible to have one animal serve as its own control.

Finally, as noted above, this type of study aims primarily towards increasing the molecular specificity of the analytical measurement of endogenous peptide contents of various biological tissues and fluid. Further studies will involve a dynamic

measurement of both the production of the enkephalins from their precursors and the inactive metabolites.

ACKNOWLEDGEMENTS

The authors acknowledge financial assistance from NIH (GM 26666 and 28611), artistic assistance of Genevieve Fridland, and typing assistance of Donna Stallings.

REFERENCES

1 R. Burgus, T. F. Dunn, D. M. Desiderio, D. N. Ward, W. Vale and R. Guillemin, *Nature (London)*, 226 (1970) 321.
2 J. Spiess, J. E. Rivier, J. A. Rodkey, C. D. Bennett and W. Vale, *Proc. Nat. Acad. Sci. U.S.A.*, 76 (1979) 2974.
3 H. Teschemacher, K. E. Opheim, B. M. Cox and A. Goldstein, *Life Sci.*, 16 (1975) 1771.
4 H. Matsuo, Y. Baba, R. M. G. Nair, A. Arimura and A. V. Schally, *Biochem. Biophys. Res. Commun.*, 43 (1971) 1334.
5 J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan and H. Morris, *Nature (London)*, 258 (1975) 577.
6 J. Hughes, *Brit. Med. Bull.*, 39 (1983) 17.
7 M. Comb, P. H. Seeburg, J. Adelman, L. Eiden and E. Herbert, *Nature (London)*, 295 (1982) 663.
8 D. L. Kirkpatrick, T. Taniguchi, B. N. Jones, A. S. Stern, J. E. Shively, J. Hulliha, S. Kimura, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.A.*, 78 (1981) 3265.
9 U. Gubler, P. Seeburg, B. J. Hoffman, L. P. Gage, and S. Udenfriend, *Nature (London)*, 295 (1982) 206.
10 M. Noda, Y. Furutani, H. Takahashi, M. Toyosato, T. Hirose, S. Inayama, S. Nakanishi and S. Numa, *Nature (London)*, 295 (1982) 202.
11 J. C. Schwartz, B. Malfroy and S. de la Baum, *Life Sci.*, 29 (1981) 1715.
12 L. E. Geary, K. S. Wiley, W. L. Scott and M. L. Cohen, *J. Pharmacol. Exp. Ther.*, 221 (1982) 104.
13 V. Y. H. Hook, L. E. Eiden and M. J. Brownstein, *Nature (London)*, 295 (1982) 341.
14 L. B. Hersch, *Biochemistry*, 20 (1981) 2345.
15 G. W. Wagner, M. A. Tavianini, K. M. Herrmann and J. E. Dixon, *Biochemistry*, 20 (1981) 3884.
16 J. Morley, *Life Sci.*, 30 (1982) 479.
17 K. L. Busch and R. G. Cooks, in F. W. McLafferty (Editor), *Tandem Mass Spectrometry*, Wiley, New York, 1983, pp. 11–40.
18 W. R. Mathews, J. Rokach and R. C. Murphy, *Anal. Biochem.*, 118 (1981) 96.
19 F. Nyberg, A. Wahlstrom, B. Sjolund and L. Terenius, *Brain Res.*, 259 (1983) 267.
20 R. Simantov and S. H. Snyder, *Mol. Pharmacol.*, 12 (1976) 987.
21 D. M. Desiderio, H. Onishi, H. Takeshita, F. S. Tanzer, C. Wakelyn, J. A. Walker, Jr. and G. Fridland, *Neurochemistry*, submitted for publication.
22 P. Angwin and J. D. Barchas, *J. Chromatogr.*, 231 (1982) 173.
23 F. S. Tanzer, D. M. Desiderio, C. Wakelyn and J. Walker, *J. Dent. Res.*, submitted for publication.
24 D. M. Desiderio and M. Kai, *Biomed. Mass Spectrom.*, 10 (1983) 471.
25 D. M. Desiderio and S. Yamada, *Biomed. Mass Spectrom.*, 10 (1983) 358.
26 D. M. Desiderio, I. Katakuse and M. Kai, *Biomed. Mass Spectrom.*, 10 (1983) 426.
27 D. M. Desiderio, *Advan. Chromatogr.* 22 (1983) 1–36.
28 F. S. Tanzer, D. M. Desiderio and S. Yamada, in D. H. Rich and E. Gross (Editors), *Peptides: Synthesis–Structure–Function*, Pierce, Rockford, IL, 1981, p. 761.
29 S. Yamada and D. M. Desiderio, *Anal. Biochem.*, 127 (1982) 213.
30 S. Yamada and D. M. Desiderio, in M. T. W. Hearn, F. E. Regnier and C. T. Wehr (Editors), *High Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1982, p. 211.
31 H. E. May, F. S. Tanzer, G. H. Fridland, C. Wakelyn and D. M. Desiderio, *J. Liquid Chromatogr.*, 239 (1982) 87.

32 M. Barber, R. S. Bordoli, G. V. Gardner, D. B. Gordon, R. D. Sedgwick, L. W. Tetler and A. N. Tyler, *Biochem. J.*, 197 (1981) 401.
33 W. C. Pickett and R. C. Murphy, *Anal. Biochem.*, 111 (1981) 115.
34 D. M. Desiderio and M. D. Cunningham, *J. Liquid Chromatogr.*, 4 (1981) 721.
35 D. M. Desiderio, J. Laughter, I. Katakuse, M. Kai and J. Trimble, *Comp. Enhanc. Spectros.*, in press.
36 S. Martin, C. Costello and K. Biemann, *Anal. Chem.*, 54 (1982) 2362.
37 D. M. Desiderio and I. Katakuse, *Biomed. Mass Spectrom.*, submitted for publication.
38 F. Field, *J. Phys. Chem.*, 86 (1982) 5115.
39 K. Levsen and H. Schwarz, *Mass Spectrom. Rev.*, 2 (1983) 77.
40 T. Kudo, H.-L. Chang, S. Maeda, J. Kakamae and R. Inoke, *Life Sci.*, 33 (1983) 677.
41 T. Kudo, S. Maeda, J. Nakamae, H.-L. Chang and R. Inoke, *Life Sci.*, 33 (1983) 681.
42 H. Jonsson, B. S. Middleditch and D. M. Desiderio, *Science*, 187 (1975) 1093.
43 B. J. Millard, *Quantitative Mass Spectrometry*, Heyden, London, 1978, 171 pp.
44 A. M. Lawson, C. K. Lim, W. Richmond, D. M. Samson, K. D. R. Setchell and A. C. S. Thomas, in A. W. Lawson, C. K. Lim and W. Richmond (Editors), *Current Developments in the Clinical Application of HPLC, GC, and MS*, Academic Press, London, 1980, p. 135.