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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND FIELD DESORPTION MASS SPECTROMETRIC MEASUREMENT OF PICOMOLE AMOUNTS OF ENDOGENOUS NEUROPEPTIDES IN BIOLOGIC TISSUE*

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SUMMARY

A unique combination of chromatographic separation and mass spectrometric techniques has been developed for a novel method for measurement of picomole amounts of endogenous oligopeptides in biologic tissue. High-performance liquid chromatography is utilized for rapid high-resolution separation of peptides. A new buffer system using dilute triethylamine–formic acid is utilized. The buffer system possesses excellent UV transparent properties enabling femtomole sensitivity for measurement of standard solutions of somatostatin. Use of porous polystyrene–divinylbenzene copolymer and octadecylsilyl columns facilitate retention of a peptide fraction from biologic extracts. Advantage was taken of field desorption mass spectrometric methods to eliminate chemical derivatization of peptides and to produce

* Abbreviations used: a.m.u. = atomic mass units; AUFS = absorbance units full scale; B = bradykinin (ArgProProGlyPheSerProPheArg); CSF = cerebrospinal fluid; CN = caudate nucleus; Dyn = dynorphin (TyrGlyGlyPheLeuArgArgIleArgProLysLeuLys); ehc = emitter heating current; FD-MS = field desorption mass spectrometry; GC = gas chromatography; GGPL = GlyGlyPheLeu; GGPM = GlyGlyPheMet; HAc = acetic acid; HPLC = high-performance liquid chromatography; IS = internal standard; k' = capacity ratio; LE = leu-enkephalin (TyrGlyGlyPheLeu); LRF = luteinizing hormone releasing factor (pGluHisTrpSerTyrGlyLeuArgProGlyNH$_2$); m/z = mass-to-charge ratio; MeOH = methanol; ME = met-enkephalin (TyrGlyGlyPheMet); $(M + H)^+$ = Protonated molecular ion; MS = mass spectrometry; MW = molecular weight; ODS = octadecylsilyl; P = substance P (ArgProLysProGlnGlnPheGlyLeuMetNH$_2$); P–D (polystyrene–divinylbenzene copolymers; ppt.. = precipitate; RIA = radioimmunoassay; RP = reversed phase; SS = somatostatin (AlaGlyCysLysAsnPhePheTrpLysThrPheThrSerCys); TEAF = triethylamine formate; TFA = trifluoroacetic acid; UV = ultraviolet; TRF = thyrotropin releasing factor; Tris = tris(hydroxymethyl)aminomethane.

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protonated molecular ions which retain total molecular information of the peptide. Use of appropriate internal standards and selected ion monitoring methods enabled nanogram sensitivity and, more importantly, optimized structural specificity of the compound being quantified. Results are compatible with radioimmunoassay data. Data obtained with field desorption mass spectrometry provide, for the first time, measurement of intact, chemically underivatized oligopeptides extracted from biologic matrices and, significantly, provide an analytic method to calibrate radioimmunoassay data. This novel combination of methods is being applied to measurement of peptides (leu-enkephalin, met-enkephalin, somatostatin, etc.) in canine brain regions and dental pulp tissue.

INTRODUCTION

An effective and efficient system has been developed to obtain biologic tissue, extract endogenous neuropeptides, and separate and measure endogenous amounts of neuropeptides with HPLC. FD-MS data provide unambiguous measurement and identification of the intact molecular structure of brain neuropeptides[1].

Development of RP-HPLC columns, techniques and buffers significantly advanced biologic peptide research methods by optimizing speed of separation, resolution and sensitivity of detection. Most peptide research utilizes ion-pairing buffers such as trialkylammonium phosphate buffers to optimize speed of separation, resolution of closely eluting peaks and sensitivity of detection[2–5]. Non-volatile buffer salts, however, interfere with RIA, MS, or biologic measurement methods. Prompted by our need of MS measurements, a volatile buffer was developed recently[6] using dilute TEAF. Pyridine-based buffers are an initial choice, but strong UV absorbance at 200 nm obviates use of this compound for this research.

One of the objectives of our research is to minimize, or if possible, avoid chemical derivatization of the peptide to minimize manipulations of limited amounts of biologic samples. While most UV monitoring of protein eluants is performed at 280 nm, this wavelength may not be appropriate for all peptides because the probability that Trp, Tyr or Phe is present decreases as peptide chain length decreases.

Bennet *et al.*[7] and Fasco *et al.*[8] published data utilizing pre-packaged mini-columns containing approximately one-half gram ODS. These columns are reusable, conveniently fit any Leuer-lock syringe and serve several purposes: (1) volume of a peptide fraction collected from HPLC is rapidly reduced by one passage through an ODS mini-column —peptides are retained on the ODS and eluted with organic solvent; (2) solutions are conveniently desalted; (3) a ODS mini-column efficiently retains neuropeptides studied and can be effectively used after protein precipitation to concentrate the peptide fraction originally extracted from tissue.

P–D columns reportedly provide a hydrophobic micro-environment with particular selectivity towards phenylalanine-containing peptides[9]. The manufacturer lists the MW operating range of these columns for peptides as 600–14,000 daltons. Peralta *et al.*[9] used P–D columns in a study of ME with a combination of enzymatic and GC–MS methods. Enkephalins contain one Phe residue and are retained efficiently on a P–D column.

A neuropeptide (oligopeptide, protein) possesses two structural features ger-

mane to RP chromatography. On one hand, a neuropeptide contains multiple ionization sites. For example, depending on the pH of the solution, unblocked N- and C-termini, Arg, Lys, Glu and Asp residues may be protonated, partially protonated or unprotonated. The concentration of each form for all ionizable residues is calculated from individual pK_a values. Because the pH operating range of ODS is below pH 8 to avoid ester bond hydrolysis and because carboxylate groups will be protonated at acid pH values, the normal buffering value utilized is *ca.* 3.

Ion pairing between peptide and buffer salts also participates in increasing hydrophobicity[10]. For example, LE in the zwitterionic form and TEAF buffer interact as follows:

$^{+}NH_3$-Tyr-Gly-Gly-Phe-Leu-COO^{-}

$HCOO^{-}$ $(CH_3CH_2)_3\overset{+}{N}H$

to minimize polarity of a peptide zwitterion, increase hydrophobicity of the peptide–TEAF ion-pair, and increase effective hydrophobic interactions between LE–TEAF and the C_{18} side chain of the ODS HPLC analytic column. In this manner, resolution of peptides of closely related structure is achieved. Stereoisomers may be separated by RP-HPLC[11]. Altering one residue (such as ^{2}Ala replacing ^{2}Gly in LE) increases hydrophobicity of the complex and increases retention time by 5 min[12]. Perfluoroalkanoic acids serve as lipophilic ion-pairing agents and play a role in hydrophobicity[13]. Integrals of individual amino acid Rekker fragmental hydrophobic indices were correlated with retention time[5] in a fashion parallel to GC retention time of oligopeptide-derived TMS perfluorinated dideuteroalkyl polyamino alcohols[14]. These data suggest that peptide, anion and cation are factors to be considered in elucidating hydrophobic effects in RP-HPLC.

While MS techniques might not be chosen *a priori* to obtain the amino acid sequence of a peptide isolated from natural sources, experience in a limited number of laboratories over the past two decades[14–17] has shown no other technique is as amenable for sequence determination when several commonly occurring situations prevail: only micrograms of material are available; blocked N- and/or C-termini; enzyme-inhibiting residues (Pro) present; all preceding criteria occur. Sequencing of the first hypothalamic releasing factor (TRF) exemplifies the last situation[15,18].

Peptide polarity results from several factors including zwitterions, interchain hydrogen bonding and polar side chains, and is reduced with appropriate chemical derivatization such as the commonly employed acetylation–permethylation reaction[19] to provide a derivatized peptide amenable to volatilization for electron or chemical ionization MS.

Recent instrumental developments and commercial availability of mass spectrometers capable of FD have recently unfolded new avenues of peptide research. Mass spectrometers capable of high resolution mass analysis of ions up to 2000–3000 a.m.u. have been of particular significance[5,20–23]. The singular capability of FD-MS in neuropeptide research is the ability to form only protonated $(M + H)^{+}$ or cationized $(M + Na)^{+}$ molecular ions. These ions contain minimal excess internal energy after ionization and little or no peptide bond fragmentation results. FD-MS yields a mass for the molecular ion which correlates with molecular structure. In-

tegrating ion current from this mass by selected ion monitoring techniques provides sensitivity at the nanogram (usually picomole) or lower level for unambiguous measurement of endogenous amounts of neuropeptides in biologic tissues and fluids[1].

ISs appropriate for FD-MS measurements include commercially available homologues (^{2}Ala–LE) or custom-synthesized peptides incorporating stable isotopes (d_7-LE, d_7-ME). Both types of IS are used for FD-MS while HPLC can utilize only the former.

Once the amino acid sequence of the first hypothalamic releasing factor was obtained by MS[15,18], other brain peptide structures were elucidated including LRF, SS, LE, ME and others. Bioassay and RIA methods were developed[24] and applied to regional localization of brain peptides[25]. Picogram amounts of individual peptides were measured with RIA. However, it was soon discovered that RIA activity cannot always be associated with only the one peptide presumably being quantified. For example, a 50,000 dalton precursor containing seven intact sequences of ME and one of LE was found[26,27]. The precursor is enzymatically cleaved to produce an intermediate peptide, which, upon further enzymolysis, produced ME and LE. Each precursor molecule exhibits enkephalin-like RIA activity. Other neuropeptides (SS) have been found to emanate from larger precursors[28]. These recent findings suggest that the following metabolic regulatory pathway may operate for these neuropeptides: a larger precursor molecule is cleaved and regulated by appropriate enzymes to produce either active peptide, or in some cases, an intermediate-sized peptide; the latter is also under enzymatic regulation. The entire cascade may be under higher order metabolic control.

An analytic scheme has been developed to separate, purify and measure each constituent in this increasingly complex metabolic scheme:

(1) Acquire nerve tissue in a rapid manner to minimize endogenous peptide loss.

(2) Efficient extraction of cytosolic neuropeptides.

(3) Fast, facile and efficient chromatographic separation of a fraction containing all putative neuropeptides.

(4) Unambiguous measurement of endogenous amounts of neuropeptides utilizing HPLC, FD-MS, IS and nominal mass.

MATERIALS AND METHODS

Tissue procurement

Dogs under pentobarbital anesthesia were exsanguinated via the left femoral artery. Brain and teeth are removed and the latter immediately placed in liquid nitrogen. Discrete brain regions including caudate nuclei, hippocampus, pituitary, cerebellum, hypothalamus, cortex, spinal cord, olfactorum tubercule, thalamus, etc. are excised and immersed in liquid nitrogen within 4 min. Samples are stored at −70°C. Tissue weights are wet weight. The weight of one pair of canine caudate nuclei is *ca.* 1 g.

Sample preparation

Samples are homogenized in HAc. Samples are defrosted, mixed with 5 ml 1.0 *N* HAc, and homogenized for 3 min at 0°C with a VirTis 23 homogenizer (Gardiner,

New York, NY, U.S.A.). Cells in this solution are disrupted for 3 min with a Kontes (Evanston, IL, U.S.A.) ultrasonic generator (300 W) with a 4 1/2 in. probe.

Internal standard and spiking

A 200-ng amount of ^{2}Ala–LE (Bachem, Torrance, CA, U.S.A.) in 20 μl Tris buffer (pH 7.4) per gram of tissue is added to the homogenization flask. In one set of experiments, a 200 ng per g tissue "spike" of LE was added.

Tracer

Homogenate is diluted ten-fold with either HAc or acetone–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.

Synthetic peptides

Other peptides were purchased from Sigma (St. Louis, MO, U.S.A.).

Protein precipitation

Proteins were precipitated with 10 volumes of acetone–0.01 *N* HCl (80:20) and removed after centrifugation at 27,000 *g*, 20 min, 0–5°C (Beckman J-21, Palo Alto, CA, U.S.A.). Supernatant was removed, five volumes of acetone–0.01 *N* HCl (60:40) added, and the sample was recentrifuged[29]. Supernatant was removed and evaporated in a nitrogen stream. Residue is centrifuged at 1090 *g* for 5 min.

Porous P–D columns

Sample was dissolved in 4 ml 10 m*M* Tris HCl buffer (pH 7.5) and placed on a P–D column (2.5 g Bio-Beads SM2, 20–50 mesh; Bio-Rad Labs., Richmond, CA, U.S.A.). A peptide fraction is eluted with 8 ml MeOH. Other workers[9] used 0.9 ml.

ODS mini-columns

ODS mini-columns are commercially available (Waters Assoc., Milford, MA, U.S.A.) pre-packaged ODS-derivatized siliconaceous columns (0.4 g) used for RP chromatography[7]. ODS mini-columns are prepared before each use by washing with 4 ml MeOH, 4 ml water, 4 ml MeOH, then 8 ml 0.5% TFA[7,8]. Sample is dissolved in 4 ml 0.5% TFA and placed on the mini-column. A peptide fraction is eluted with 2 ml acetonitrile–0.1% TFA (80:20).

Radioactivity counting

Radiolabeled LE is traced by counting radioactivity (Hewlett-Packard Tri-Carb 460C) after protein precipitation, before and after P–D and ODS columns, and after HPLC separation.

HPLC

A Waters Assoc. HPLC apparatus was used and consisted of a U6K injector, guard column packed with Corasil B (50 μm particle diameter), μBondapak C$_{18}$ (10 μm particle diameter, RP analytical stainless-steel column, 30 cm × 4 mm), two Model 6000A solvent delivery pumps, Model 600 solvent programmer and a Model

450 variable-wavelength UV detector[5]. Analogue signals were recorded on a Houston recorder or Waters Data Module; the latter provides integrated areas in arbitrary units. HPLC resolution is conveniently increased by coupling two or more HPLC columns in series.

A novel volatile buffer was used for peptide resolution. Dilute formic acid (0.04 *M*) was titrated to pH 3.15 with distilled triethylamine to form TEAF buffer[6,30]. Aqueous solutions were filtered and degassed with Millipore filters (0.45 μm pore diameter, HAWP 04700; Millipore, Bedford, MA, U.S.A.) as were organic solutions (FHIP 04700, 0.5 μm pore diameter).

Samples were dissolved in TEAF (100 μl) and, after HPLC resolution into individual peaks, fractions collected by switching the "waste-recycle-collect" valve to "collect". Volume of collected solvent (several milliliters) was reduced by elution through a Sep-Pak.

FD–MS

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 was used. Nominal resolution was 1000; source temperature 90°C; emitter potential +8 kV; counter electrode −3 kV. Emitters were fabricated in our laboratory from 10 μm diameter tungsten wire and activated at high temperature in a benzonitrile atmosphere with a high electric field. Carbon microneedle growth on the emitter wire surface extended to a length of approximately 30 μm.

Sample is dissolved in 100 μl MeOH to wash the sides of the reactivial and a gentle stream of nitrogen reduces sample volume to less than 10 μl. Sample is transferred to the emitter with a microsyringe–micromanipulator–stereomicroscope ensemble[1]. Non-silanized glassware is used whenever FD-MS measurements are obtained.

The peak-matching unit scans alternately $(M + H)^+$ ions of LE at m/z 556 and ^{2}Ala-LE at 570. Emitter heating current is increased manually to 16 mA where peptides desorb optimally[5] and an oscillographic recording is obtained manually of the entire desorption envelope as peak switching continues. Individual ion currents of the two $(M + H)^+$ ions are integrated manually. The FD-integrated ion current of LE is 0.75 times the current due to IS for equal amounts of sample placed on the FD emitter.

RESULTS AND DISCUSSION

Recovery studies

Fig. 1 lists methods used for tissue homogenization, protein precipitation, lipid extraction and chromatography using ODS and P–D columns. Percentages listed to the left indicate amount of radioactivity due to exogenous tritiated LE. Tritiated LE is added after homogenization and ultrasonication but before precipitation of proteins with acidified acetone. Ten per cent of the radioactivity is lost after protein precipitation. After overnight equilibration, centrifugation and removal of acetone with nitrogen, lipids are removed with two-fold extraction with ether. Minimal radioactivity (3%) is lost during this step. The resulting fraction contains mainly

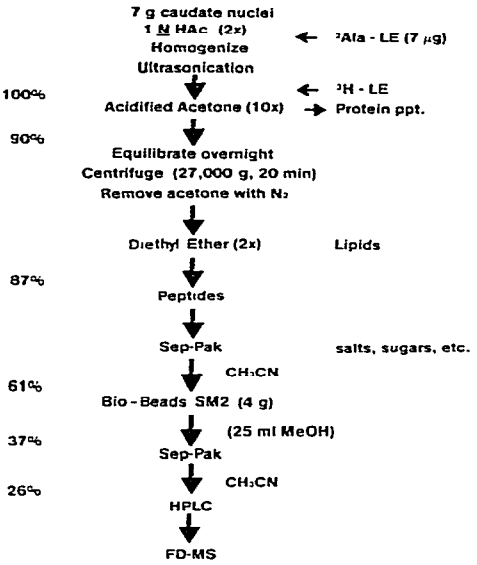

Fig. 1. Flow chart illustrating sample preparation steps, ODS, P–D, HPLC, % recovered radioactivity and amounts.

peptides but also salts, sugars and other polar material. Peptides are retained on an ODS mini-column after elution. Eighty per cent acetonitrile elutes 84% of the peptide fraction from the mini-column. This loss reflects a biologic matrix effect because nearly quantitative (95–97%) recovery of radioactivity occurs after eluting a synthetic peptide from an ODS minicolumn. The peptide fraction is eluted from a P–D column (4 g) where 37% of the original amount of radioactivity remains after elution of the peptide fraction with methanol. After desalting and volume reduction with a final ODS mini-column, 26% of the original radioactivity remains. This peptide fraction is subjected to HPLC and collected fractions measured for endogenous levels of enkephalins with FD-MS. An ODS mini-column is required before and after Bio-Beads columns with gram amounts of brain tissue to remove an interfering HPLC peak eluting with 30% acetonitrile. Milligram amounts of dental tissue do not require use of ODS mini-columns.

P–D column chromatography

Peralta *et al.*[9] utilized P–D columns for measurement of ME and found 75–80% of tritiated ME was recovered utilizing 80 ng synthetic ME with 0.9 ml MeOH.

Vogel and Altstein[31] used Porapak Q (copolymer of styrene and ethylvinylstyrene cross-linked with divinylbenzene) to adsorb enkephalins. A 80-mg amount of Porapak Q was washed with ethanol, 50% aqueous ethanol, then two times with either Tris buffer or sodium phosphate at pH 7.5. These authors concluded that a phenylalanine residue is very important for retention of enkephalins on Porapak Q columns. LE and ME were quantitatively absorbed on a Porapak Q column from a Tris buffer, then eluted with MeOH.

In our study, 2.5 g of P–D was the amount of required column material and

was derived empirically for a column where 90% radioactivity was recovered with elution of up to 10 ml MeOH. The remaining 10% of radioactivity was recovered with elution of up to 40 ml of MeOH. We found it beneficial to equilibrate P–D column material overnight in ethanol before use. Optimal results for biologic samples are obtained with a 1:2 ratio of P–D column material to tissue. This two-fold ratio is required due to biologic matrix effects. Vogel and Altstein[31] refer to a 1:10 absorption capacity of Porapak Q columns for enkephalins. We note without comment at this time, however, that substance P, which contains two Phe residues, is unretained on a P–D column.

HPLC of standards

Fig. 2 contains the HPLC chromatogram of a standard mixture of seven synthetic peptides: dynorphin[32], GGPM, B, GGPL, ME, P and LE. The two tetrapeptides are included to indicate where potential enkephalin metabolites elute. Enzymatic activity of tissue homogenates results in cleavage of the susceptible Tyr–Gly bond in ME and LE[31]. Two pairs of peptides (GGPL/ME and P/LE) are unresolved at this percentage organic modifier. There is no difficulty in altering experimental conditions to resolve these pairs. Controllable parameters include type of buffer, pH, flow-rate, ionic strength, type and percentage of organic modifier, temperature, type of column packing, use of recycle mode and number of columns in series[2]. However, complete resolution of a synthetic mixture of peptides is not the objective of this research. Our experience indicates the relative ease of separating any given mixture of synthetic standards, in contradistinction to separating peptides from biologic extracts. The latter extracts are much more complex and contain many more peaks due

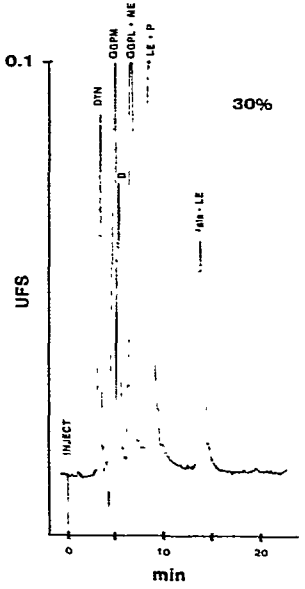

Fig. 2. HPLC chromatogram of a mixture of seven synthetic peptides: Dyn, GGPM, B, GGPL, ME, P and LE. A 1-μg amount of each peptide was injected. Experimental conditions: 70% TEAF buffer, pH 3.15, 200 nm, 1.5 ml/min, 0.1 AUFS, 60 cm × 4 mm, μBondapak C_{18} columns, recorder paper speed 0.5 cm/min.

to peptides, non-peptide compounds, metabolites, precursors, among other compounds. These constituents contribute to the oft-mentioned, yet ill-defined, "biologic matrix effect".

Therefore, one of the basic objectives for analysis of biologic samples remains: the degree of chromatographic resolution of endogenous compounds is reflected by the molecular specificity of the subsequent analytic method. (This statement parallels the standard relationship in spectroscopy: sensitivity × resolution = a constant). Our experience indicates that the separation scheme presented above provides a significant concentration of peptides following separation from other compounds with brain and dental pulp peptides.

As mentioned above, less chromatography is needed for dental pulp extracts. One cannot rigorously guarantee that one HPLC peak is only one compound even after multiple chromatographic steps. If the molecular specificity of detection is not as high as it might be, the only remaining choice is to continuously apply different columns, solvent, etc., to the separation scheme in the hope of establishing purity.

On the other hand, MS detection and measurement provide maximum molecular specificity with minimal interference. FD-MS methods can now be applied to underivatized intact, biologic peptides[1,20,22,23]. Therefore, HPLC separation can be optimized[5] (which may or may not be equivalent to maximized) and measurement of endogenous amounts of peptide guaranteed by FD-MS[1].

RP-HPLC retention time is not a function only of hydrophobic fragmental indices. For example, Rekker integrals of LE (5.93) and substance P (9.01) differ significantly, yet these two peptides coelute at 30% acetonitrile. However, other parameters may play a role in the RP-HPLC behavior of this pair of peptides. At 20% acetonitrile, a more hydrophobic micro-environment compared to 30%, the pentapeptide LE elutes (8.6 min) while the undecapeptide substance P does not, indicating that peptide chain length may be a potential factor.

HPLC of pulp tissue

Fig. 3 contains the HPLC chromatogram of the peptide fraction extracted from dental pulp tissue (444 mg) from one animal. Based on peak area ratios of LE (8.2 min) and ^{2}Ala-LE (13.6 min), amount of endogenous LE equals:

$$\frac{\text{peak area LE (68)}}{\text{peak area } {}^2\text{Ala-LE (20)}} \times \text{amount added } {}^2\text{Ala-LE (2)} =$$

$$3.4 \times 2\ \mu\text{g/g} = 6.8\ \mu\text{g/g pulp tissue}$$

From HPLC data alone, this number represents an accurate measurement of endogenous LE using an internal standard because both HPLC peaks are visually well resolved from neighbors. Indeed, many laboratories measure endogenous compounds in this way. Nevertheless, HPLC data must be substantiated with an FD-MS measurement. Amount of LE measured by HPLC (6.8 μg LE/g tissue) is one order of magnitude higher than RIA measurements of LE in caudate nuclei[33].

It is interesting to note that more than a dozen peaks occur in this chromatogram. Some peaks co-chromatograph with known peptides, but most do not. For example, substance P would elute slightly following LE at 8 min. The presence of these peaks suggests a working hypothesis: each peak may represent an endogenous, poten-

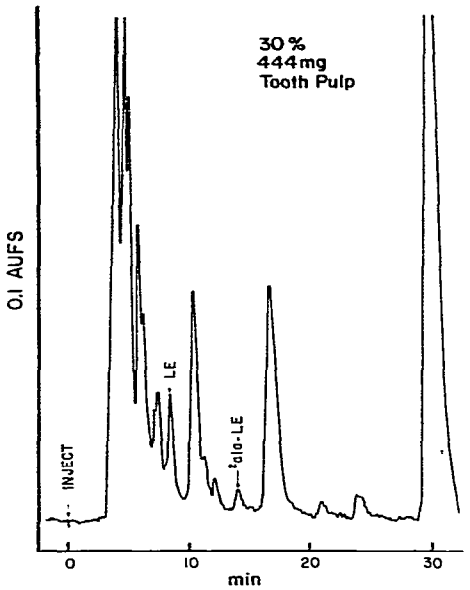

Fig. 3. HPLC chromatogram of peptide fraction extracted from 444 mg canine tooth pulp. See Fig. 2 for experimental conditions.

tially bioactive peptide in this biologic extract. This hypothesis is supported by several circumstantial pieces of experimental information: proteins, glycoproteins, lipoproteins, lipids and sugars are removed during chromatography with specific solvents; specific peptide hydrophobic interactions are taken advantage of during HPLC; specific affinity of P–D and ODS mini-columns towards peptides; a specific wavelength is used to observe peptide-bond UV absorption; isocratic elution avoids common gradient elution artifacts and optimizes retention time reproducibility. Further structural proof underway in our laboratory will prove or disprove this hypothesis.

HPLC of caudate nuclei

A known weight (5 g) of CN tissue plus IS (10 μg) was divided into two equal parts and a LE spike added to one portion. Each portion was subjected to ODS, P–D and HPLC chromatography. Fig. 4 contains the HPLC of the unspiked sample while Fig. 5 contains the HPLC of the spiked sample.

Data from our laboratory indicate different chromatographic recoveries for LE *versus* ^{2}Ala-LE using ODS, P–D and HPLC columns. For example, Table I collects data for recoveries following ODS and P–D columns for LE and ^{2}Ala-LE.

TABLE I

RECOVERIES OF LE AND ^{2}Ala-LE FOLLOWING ODS AND P–D COLUMNS

Column	*Recovery (%)*	
	LE	*^{2}Ala-LE*
ODS	72	56
P–D	90	86

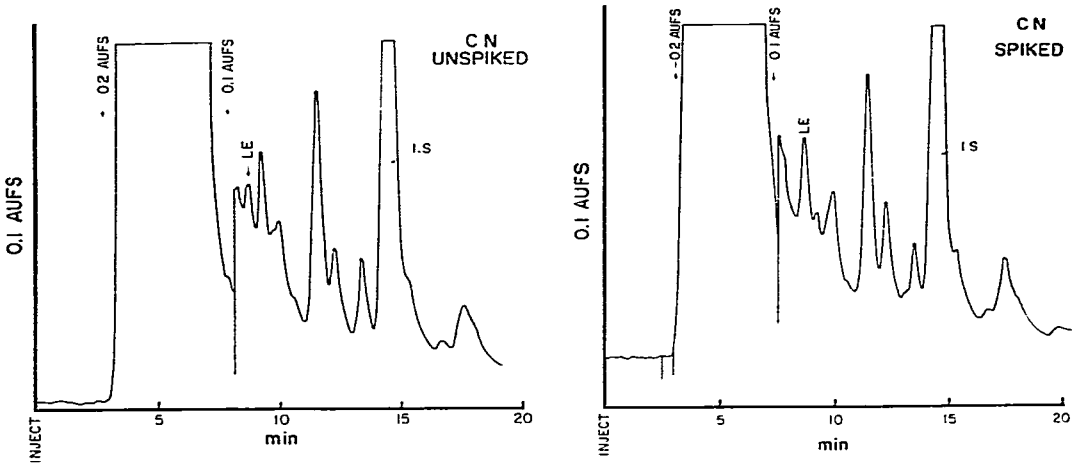

Fig. 4. HPLC chromatogram of peptide fraction extracted from 2.5 g canine caudate nuclei. See Fig. 2 for experimental conditions.

Fig. 5. HPLC chromatogram of peptide fraction extracted from 2.5 g caudate nuclei spiked with 200 ng LE per g tissue. See Fig. 2 for experimental conditions.

These data are obtained by comparing HPLC areas (peak height times width at half-height) before and after elution from an ODS or P–D column. This information is generally significant for any analysis using an IS which has a different partition coefficient and separates chromatographically during multiple purification steps. For example, CN tissue is subjected to ODS, P–D, then a second ODS column. If 100 μg LE were applied, then 47 μg LE (100 × 0.72 × 0.90 × 0.72) and 27 μg ^{2}Ala-LE (100 × 0.56 × 0.86 × 0.56) are collected. A correction factor (42/27 = 1.74) must be applied to the final measurement.

On the other hand, the set of two equations with two unknowns compensate for differential chromatographic behavior of IS[34].

TABLE II

HPLC AREAS (ARBITRARY UNITS) OF LE AND IS FOR SPIKED AND UNSPIKED SAMPLES OBTAINED FROM DATA MODULE

Ratio (R) equals area due to LE/area due to IS.

	Unspiked	*Spiked*
LE	4257	7807
IS	36,372	23,763
R	0.117 (R_1)	0.329 (R_2)

The following two equations and two unknowns are used and compensate for differential recoveries of LE and IS. For unspiked sample:

$$ax = R_1 \quad (1)$$

where x = amount endogenous LE, R_1 = ratio of LE/IS area, $a = R_1/x$

For spiked sample

$$a\,(x + 500) = R_2 \tag{2}$$

or

$$ax + 500a = R_2 \tag{3}$$

Substituting eqn. 1 in eqn. 3,

$$R_1 + 500a = R_2 \tag{4}$$

$$a = (R_2 - R_1)/500 \tag{5}$$

Substituting eqn. 5 in eqn. 1 and solving for x yields

$$x = \frac{500\ R_1}{R_2 - R_1} \tag{6}$$

Substituting values for R_1 and R_2 in eqn. 6 yields

$$x = \frac{(500)\ (0.117)}{(0.329 - 0.117)} = 276 \text{ ng LE/2.5 g CN, or, } 110 \text{ ng LE/g CN}$$

FD-MS measurement of endogenous LE in CN tissues

In a separate experiment using other CN tissue, an amount (7 g) of CN tissue was extracted and subjected to ODS mini-column, P–D and HPLC chromatography. Eluents of HPLC peaks were collected in one vial and only at appropriate LE and IS retention times. Duplicate FD-MS measurements on this collected sample yielded ratios of integrated ion current due to LE/IS of 0.34 and 0.37 with an average of 0.355. Endogenous amount of LE equals

$$\frac{0.355 \times 7}{1.74 \times 0.75} = 1.90\ \mu\text{g LE/7 g CN} = 272\ \text{ng/g CN}$$

where 0.75 is an empirical factor correcting for FD response of LE *versus* IS and 1.74 is the correction factor for differential chromatographic behavior of IS *versus* LE. The latter factor must be used when a deuterated IS is not used.

This value (272 ng LE/g CN) represents, in our opinion, the most accurate measurement of LE in a biologic tissue and is the first measurement made with this method. Improvements in the method are already underway. For example, a deuterated IS (d_7-Tyr-LE) is being synthesized. This deuterated IS will (1) possess more equivalent HPLC elution and FD-MS desorption properties, (2) avoid the two correction factors noted above and (3) increase accuracy of FD-MS measurements.

CONCLUSIONS

Fresh brains were obtained to optimize recovery of endogenous neuropeptides by avoiding further biosynthesis and degradation of neuropeptides. Microsurgical techniques ensure excision of specific brain regions and subsequent measurement of only the neuropeptide content of an individual brain region.

RP-HPLC utilizing ODS analytic columns is well suited to separation of neuropeptides from biologic tissue[5,20,35]. Recovery of an injected peptide is nearly quantitative[2], resolution of close k' values is high[5], and speed of separation is minutes because isocratic conditions can be adjusted to elute any peptide at any HPLC retention time. The last statement is not strictly the case for biologic samples where more polar species must be eluted first to ensure baseline has been attained before elution of compounds of interest to optimize accuracy of HPLC measurement.

Sensitivity of the variable-wavelength UV detector towards peptides is high[6]. Femtomoles of synthetic solutions of SS are detected. Furthermore, recent commercially available UV detectors indicate a 10-fold increase in sensitivity. A variable-wavelength UV monitor for detection of peptides is recommended for this type of peptide research where advantage is taken of the very high molar absorptivity of the peptide bond in the 190–210 nm range as opposed to the 280 nm wavelength commonly employed[36] (molar absorptivity at 197 nm of ME = 10^5). The latter wavelength does not detect peptides when no aromatic residue occurs, while the former is a universal wavelength to detect all peptide bonds.

Separation of individual peptides appears to follow increasing hydrophobicity, specifically hydrophobic interactions between peptide–buffer complex and ODS chains of the analytic column packing[10]. Peptides are eluted from the ODS packing by either increasing percentage of organic modifier during gradient elution or by an increased retention time under isocratic conditions.

The most important HPLC parameter in a study of biologic neuropeptides is reproducibility of retention time of elution. No difficulties are observed in reproducibility of retention time with pure standards[5] where coefficient of variation of retention time of multiple injections is approximately 4%. Dependability on coefficient of variation is important when working with biologic samples because a specific peptide fraction is collected manually at a retention time determined previously with standards.

The TEAF HPLC buffer is compatible with FD-MS for measurement of endogenous amounts of neuropeptides from brain and dental pulp tissue. Sensitivity with the TEAF buffer system regularly attains the picomole level for biologic samples and the fmol level for standards. Lyophilizability of TEAF enables direct, subsequent RIA, bioassay or FD-MS measurements.

Table III collects data from HPLC and FD-MS measurements of endogenous amounts of LE in CN tissue.

Experience accumulated in our laboratory suggests that caution be employed in interpreting each value listed in Table I. On one hand, potentially unresolved peptides or other compounds may coelute on HPLC with either LE or IS. On the other hand, the algorithm for calculation of unresolved or overlapping HPLC peaks must be known exactly otherwise an area of unknown accuracy is calculated by a data module.

TABLE III

ENDOGENOUS AMOUNTS OF LE

Based on 10% protein in wet tissue[40].

Measurement	*Amount of LE (ng/g CN)*
HPLC	110
FD–MS	272
RIA	520 (37)
	200 (33)
	64 (38)
	14 (39)

In principle, FD-MS data are the most accurate measurements. Nonetheless, in this first report discussing use of this technique, it is recognized that two problems exist, and indeed, are currently being investigated. On one hand, IS should not separate from LE during any chromatographic step.

A higher homologue of a peptide is not an ideal IS. An extra methyl group increases hydrophobicity of the LE molecule. A more trivial method to be solved is to take a larger amount of CN and divide it into two equal portions, followed by FD-MS measurement. This comparison will indicate precision of the method.

A further increase in accuracy and precision of the FD-MS measurement will be provided by microprocessor control of several steps in the measurement including reproducible emitter heating current increase, maintainment of a constant peptide desorption profile, acquisition of individual ion currents and ion current signal accumulation. Microprocessor circuitry is currently being fabricated in our laboratory for these purposes.

FD-MS measurement of collected HPLC neuropeptide fractions substantially increases confidence that the substance being quantified is the only structure one is dealing with[1,41]. Although this statement appears obvious and trivial at first, this fact is not always guaranteed when quantifying endogenous neuropeptides (or any other compound) by either a chromatographic technique or RIA only. In the first case, an UV absorption only can be attached to a peak eluting at a specific retention time, while in the second case a radioimmunoassayable portion of the molecule is generally what is actually being quantified. However, a unique parameter, nominal mass, is attached to a singular HPLC retention time for a neuropeptide measured with FD-MS. The authors realize that in some cases a further increase of specificity is required and in those cases, either accurate mass determination or the novel technique of collision activation MS[42] verifies that the structure being quantified is the structure of interest.

RIA measurements will not be replaced by FD-MS. RIA offers great advantages of speed, sensitivity and cost. Nonetheless, it is possible for the first time to calibrate RIA with FD-MS for peptide measurements.

HPLC and FD-MS methods described in this paper are being applied to measurement of endogenous amounts of individual neuropeptides in dental pulp tissue and discrete brain regions.

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REFERENCES

1 D. M. Desiderio, S. Yamada, J. Z. Sabbatini and F. Tanzer, *Biomed. Mass Spectrom.*, 8 (1981) 10.
2 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343.
3 J. Rivier, R. Wolbers and R. Burgus, in M. Goodman and J. Meienhofer (Editors), *Peptides — Proceedings of the Fifth American Peptide Symposium*, Wiley, New York, 1980, p. 52.
4 W. S. Hancock, C. A. Bishop, J. E. Battersby, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 168 (1979) 377.
5 D. M. Desiderio, J. L. Stein, M. D. Cunningham and J. Z. Sabbatini, *J. Chromatogr.*, 195 (1980) 369.
6 D. M. Desiderio and M. D. Cunningham, *J. Liquid Chromatogr.*, 4 (1981) 721.
7 H. P. J. Bennett, C. A. Browne, P. L. Brubaker and S. Solomon, in G. Hawk (Editor), *Proc. 3rd Int. Liquid Chromatography Symp.*, in press.
8 M. J. Fasco, M. J. Cashin and L. S. Kaminsky, *J. Liquid Chromatogr.*, 2 (1979) 565.
9 E. Peralta, H.-Y. T. Yang, J. Hong and E. Costa, *J. Chromatogr.*, 190 (1980) 43.
10 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
11 B. Larsen, B. L. Fox, M. F. Burke and V. J. Hruby, *Int. J. Pept. Prot. Res.*, 13 (1979) 12.
12 D. M. Desiderio, unpublished results.
13 W. M. M. Schaaper, D. Voskamp and C. Olieman, *J. Chromatogr.*, 195 (1980) 181.
14 H. Nau and K. Biemann, *Anal. Biochem.*, 73 (1976) 139.
15 R. Burgus, T. F. Dunn, D. Desiderio, D. N. Ward, M. Vale and R. Guillemin, *Nature (London)*, 226 (1970) 321.
16 J. Hughes, T. W. Smith, H.-W. Kosterlitz, L. A. Fothergill, B. A. Morgan and H. R. Morris, *Nature (London)*, 258 (1975) 577.
17 M. Barber, P. Jolles, E. Vilkas and E. Lederer, *Biochem. Biophys. Res. Commun.*, 18 (1965) 469.
18 D. M. Desiderio, R. Burgus, T. F. Dunn, W. Vale, R. Guillemin and D. N. Ward, *Org. Mass Spectrom.*, 5 (1971) 221.
19 P. A. Leclercq and D. M. Desiderio, *Anal. Lett.*, 4 (1971) 305.
20 D. M. Desiderio, J. Z. Sabbatini and J. L. Stein, *Advan. Mass Spectrom.*, 8 (1980) 1298.
21 H. D. Beckey and H.-R. Schulten, *Angew. Chem., Int. Ed. Engl.*, 14 (1975) 403.
22 W. Frick, E. Barofsky, G. D. Daves, D. F. Barofsky, D. Chang and K. Folkers, *J. Amer. Chem. Soc.*, 100 (1978) 6221.
23 M. Przybylski, I. Luderwald, E. Kraas and W. Voelter, *Z. Naturforsch. B*, 34 (1979) 736.
24 V. Clements-Jones, P. J. Lowry, L. H. Rees and G. M. Besser, *Nature (London)*, 283 (1980) 295.
25 A. S. Stern, R. V. Lewis, S. Kimura, J. Rossier, L. D. Gerber, L. Brink, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 6680.
26 R. V. Lewis, A. S. Stern, S. Kimura, J. Rossier, S. Stien and S. Udenfriend, *Science*, 208 (1980) 1459.
27 B. N. Jones, A. S. Stern, R. V. Lewis, S. Kimura, S. Stein, S. Udenfriend and J. E. Shively, *Arch. Biochem. Biophys.*, 204 (1980) 392.
28 P. Brazeau, N. Ling, F. Esch, P. Bohlen, R. Benoit and R. Guillemin, *Regul. Pept.*, 1 (1981) 255.
29 H. Ueda, H. Shiomi and H. Takagi, *Brain Res.*, 198 (1980) 460.
30 D. M. Desiderio, M. D. Cunningham and J. Trimble, *J. Liquid Chromatogr.*, in press.
31 Z. Vogel and M. Altstein, *FEBS Lett.*, 80 (1977) 332.
32 A. Goldstein, S. Tachibana, L. I. Lowney, M. Hunkapiller and L. Hood, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 6666.
33 A. Dupont, J. Lepine, P. Langelier, Y. Merand, D. Rouleau, H. Vaudry, C. Gros and N. Barden, *Regul. Pept.*, 1 (1980) 43.
34 B. J. Millard, *Quantitative Mass Spectrometry*, Heyden, London, 1978.
35 H. R. Morris, A. T. Etienne, A. Dell and R. Albuquerque, *J. Neurochem.*, 34 (1980) 574.

36 A. Dell, T. Etienne, H. R. Morris, A. Beaumont, R. Burrell and J. Hughes, in I. MacIntyre and M. Szelke (Editors), *Molecular Endocrinology*, Elsevier, Amsterdam, New York, 1979, p. 91.
37 I. Lindberg and J. L. Dahl, *J. Neurochem.*, 36 (1981) 506.
38 M. J. Kubek and J. Wilber, *Neurosci. Lett.*, 18 (1980) 155.
39 P. C. Emson, A. Arregui, V. Clement-Jones, B. E. B. Sandberg and M. Rossor, *Brain Res.*, 199 (1980) 147.
40 K. Diem and C. Lentner, *Scientific Tables*, Ciba-Geigy, Basel, 1970, p. 520.
41 E. Granstrom and H. Kindahl, *Advan. Prostagl. Thrombox. Res.*, 5 (1978) 119–210.
42 D. M. Desiderio and J. Z. Sabbatini, *Biomed. Mass Spectrom.*, in press.