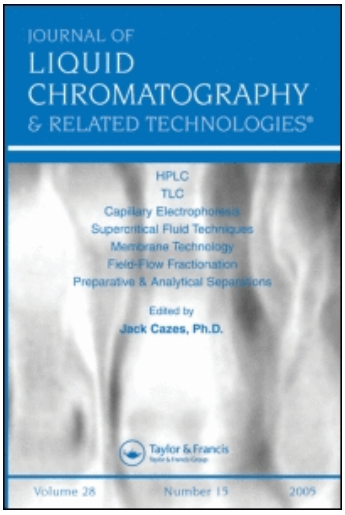

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Capillary Electrophoresis Coupled to Fluorescence Detection for the Determination of in Vivo Release of Multiple Neuropeptides from the Ewe Median Eminence

Juan P. Advis[a]; Norberto A. Guzman[bc]

[a] Department of Animal Sciences, Rutgers University, New Brunswick, New Jersey [b] Protein Research Unit, Princeton Biochemicals, Inc., Princeton, New Jersey [c] The R. W. Johnson Pharmaceutical Research Institute, a Johnson and Johnson company, Raritan, New Jersey

To cite this Article Advis, Juan P. and Guzman, Norberto A.(1993) 'Capillary Electrophoresis Coupled to Fluorescence Detection for the Determination of in Vivo Release of Multiple Neuropeptides from the Ewe Median Eminence', Journal of Liquid Chromatography & Related Technologies, 16: 9, 2129 — 2148

To link to this Article: DOI: 10.1080/10826079308019920

URL: http://dx.doi.org/10.1080/10826079308019920

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(9&10), 2129-2148 (1993)

CAPILLARY ELECTROPHORESIS COUPLED TO FLUORESCENCE DETECTION FOR THE DETERMINATION OF IN VIVO RELEASE OF MULTIPLE NEUROPEPTIDES FROM THE EWE MEDIAN EMINENCE

JUAN P. ADVIS[1] AND NORBERTO A. GUZMAN[2,3]
[1]*Department of Animal Sciences*
Rutgers University
New Brunswick, New Jersey 08903
[2]*Protein Research Unit*
Princeton Biochemicals, Inc.
Princeton, New Jersey 08543

ABSTRACT

The link between brain and reproduction is the release of the decapeptide luteinizing hormone-releasing hormone (LHRH) from the brain median eminence (ME), a release modulated by other neuropeptides such as ß-endorphine (ßEND) and neuropeptide Y (NPY) impinging on the LHRH neuron. Analysis of brain perfusates is the method of choice to determine **in vivo** release from discrete brain areas. For this, push-pull cannula (PPC) and microdialysis sampling are the most prominent technologies. However, sample volume is a limiting issue when attempting to measure by radioimmunoassays (RIA) **in vivo** release of multiple neuropeptides from these sources, especially when neuropeptide concentration is low and the whole sample

[3]Current address: The R.W. Johnson Pharmaceutical Research Institute, a Johnson and Johnson company, Raritan, New Jersey 08869.

(100 µl PPC perfusate/10 min) has to be dedicated to measure only one neuropeptide. We developed a capillary electrophoresis (CE)-based assay for the simultaneous determination of LHRH, ßEND and NPY in PPC samples from the ewe ME. This assay uses CE for the separation of these neuropeptides and their derivatization with the fluorogenic chromophore fluorescamine to amplify their signal. The CE-based assay (120 nl of PPC perfusate injected into the capillary column) allows the simultaneous electrophoretic assessment of endogenous NPY and ßEND (found at levels between 10-100 pg/100 µl PPC perfusate/10 min as resolved by RIA). However, simultaneous detection of endogenous LHRH (present at levels between 0.1-1 pg/100 µl PPC perfusate/10 min) is only marginal. A working assay for LHRH might only be achieved when laser-induced fluorescence detection of derivatized neuropeptides is coupled to additional sensitivity enhancers already in existence. An appealing aspect of this technology is the simplicity of the methodology, its efficiency coupled to the need of low volume samples, and most important, its ability to separate and to detect multiple neuropeptide components, simultaneously, in a single sample.

INTRODUCTION

Analysis of brain perfusates might become the method of choice to determine **in vivo** reactions in the brain [1-3]. For this, push-pull cannula and microdialysis sampling are the two most prominent technologies to sample **in vivo** release from discrete brain areas. The main drawback, when attempting to measure multiple neuropeptides in perfusates obtained using these sampling approaches, is the low sample volume (20-100 µl) and the low neuropeptide concentration in these samples. The latter problem is further magnified when using microdialysis, because neuropeptide recovery through its probe range from 0.5-15% [1] depending on the neuropeptide and the length of the dialysis membrane used (2 vs 5 mm in length). In addition, microdialysis probes 5 mm long cannot be used when dealing with small target areas or areas adjacent to bone structures, as is true of the median eminence in the hypothalamus. On the other hand, neither recovery of neuropeptides nor position of the tip probe are problems when PPC sampling is used at this latter site.

Radioimmunoassay is the most used technique to determine neuropeptide content in PPC samples from discrete brain areas. Currently, most RIA allows detection of **in vivo** neuropeptide re-

lease from the ME with high sensitivity and specificity. However, the large sample volume required for each RIA (10-200 µl) limits their usefulness when several neuropeptides (and neurotransmitters) need to be measured in a small sample volume (100 µl) of PPC perfusate and at small time intervals (10 min). For example, it is necessary to determine **in vivo** release of multiple neuropeptides from the ME when attempting to understand their interactions at this level in relation to the neuroendocrine control of reproduction. The LHRH neuronal system is the sole link between the brain and all reproductive-related activity. Hypophysiotropic LHRH neurons make neurohemal contacts at the ME, where they release the LHRH decapeptide to primary portal vessels. Through these vessels, LHRH reaches the anterior pituitary gland and controls the secretion of gonadotropin hormones and therefore reproduction [4-6]. Since the release of LHRH from the ME as well as the release of LH from the anterior pituitary are pulsatile, short time interval sampling (e.g., 10 min) is required to resolve their release pattern. Furthermore, it has been suggested that the hypophysiotropic output of LHRH from the ME is controlled by other neuropeptides impinging into LHRH neuronal terminals [7-9], as for example ß-endorphin and neuropeptide Y. Therefore, it is important to measure the release of these three neuropeptides in each PPC perfusate sample [10, 11].

Capillary electrophoresis, a powerful analytical and separation technique, has the potential to assess multiple neuropeptides in a small sample volume [12-14]. For example, CE has been used to measure neurotransmitters in a single cell [15], while others have reported zeptomole levels (10^{-21} mole/L) of detectability [16]. In addition, it is also possible to use femtoliters (10^{-15} L) of sampling volume [15].

The objective of the present work was to determine if CE coupled to fluorescence detection might be a viable approach to measure **in vivo** release of LHRH, ßEND, and NPY obtained from the ME by PPC sampling, during the ewe estrous cycle. For comparison purposes, UV detection of the same derivatized neuropeptide standards and PPC samples was also performed.

EXPERIMENTAL

Samples

In vivo release samples were obtained by push-pull perfusion of the posterior-lateral ME during synchronized luteal and follicular phases in ewes, using a multiple guide cannula assembly (MGCA) and remov-

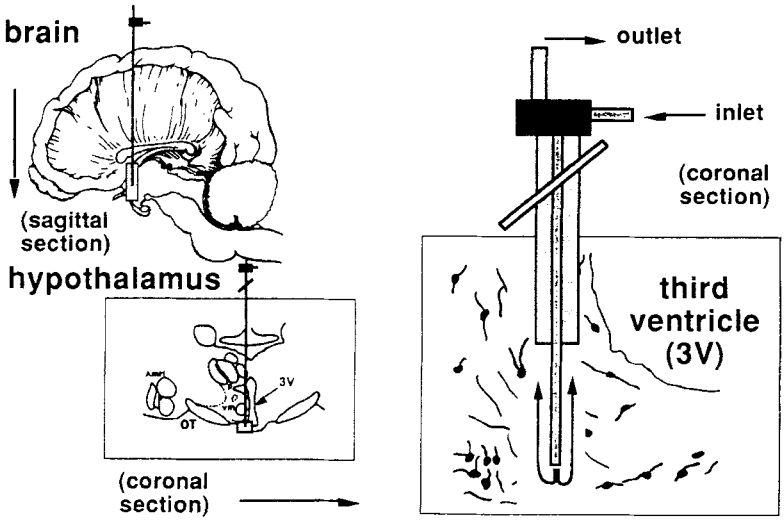

Figure 1. Diagram of the Push-Pull Cannula (PPC) Probe and of its Target Area. The PPC probes goes through the brain into the hypothalamus, the rectangle outline in the sagital view and enlarged in the coronal view below. At the center of the hypothalamus is the third ventricle of the brain. The floor of the third ventricle, outlined in this small rectangle and enlarged in the right panel, is the ME. At this latter level it is shown the third ventricle and the tip of the PPC probe. Artificial CSF is perfused through the inner tube (push) and it is withdraw at the same rate through the outer concentric tube (pull).

able push-pull cannula probes [10]. In each ewe, the MGCA was attached to the skull over the ME using stereotaxic surgery. This device allowed repetitive sampling of this discrete ME area (see Figure 1). As previously reported [10], the specific guide cannulae of the assembly (1 of 48 cannula) located on the midline directly on top of the central portion of the ME was defined during surgery, based on roentgenograms obtained after infusion of a radio-opaque dye into the third ventricle. Thus, spatial parameters were obtained to reach the sampling area with removable PPC probes. Each PPC probe was formed by two concentric stainless steel cannula (Plastic One, Roanoke, VA). The inner cannulae (28 gauge) projects 1 mm from the outer cannulae (21 gauge). This PPC probe perfused a sphere of extracellular space at the target site of approximately 1.0-1.5 mm diameter having the tip of the PPC probe at its center.

The LHRH, ßEND, and NPY content in each PPC sample was assessed using specific RIAs, as follows: for LHRH we used a published protocol [17, 18] with the Chen-Ramirez antibody provided to us kindly by Dr. Ramirez (University of Illinois, Champaign-Urbana, IL); for ßEND we used a published protocol [19] with the Y-10 antibody provided kindly by Dr. Sarkar (Washington State University, Pullman, WA); and for NPY we used a published protocol [20] with the antibody provided kindly by Dr. McDonald (Emory University, Atlanta, GA). A portion from each luteal and follicular PPC sample (usually 5-10 µl of 100 µl PPC perfusate) obtained at 10 min intervals for 4-8 hr was collected into a luteal and a follicular pool, respectively. These pools were then aliquoted into 100 µl units and stored frozen, as a continuos source of samples, for further testing by a CE-based assay.

Reagents

All chemicals were obtained at the highest purity level available from the manufacturer and were used without additional purification. Sodium hydroxide, sodium phosphate (Na_2HPO_4), lithium chloride, borax ($Na_2B_4O_7 \cdot 10H_2O$), and fluorescamine were purchased from Sigma Chemical Co (St. Louis, MO). Acetone (HPLC grade), pyridine (Fisher Certified), and hydrochloric acid solution (12 M) were obtained from Fisher Scientific (Fair Lawn, NJ). Neuropeptide standards (LHRH, NPY, ßEND) were purchased from Peninsula Laboratories (Belmont, CA). Reagent solutions and buffers were prepared using triply distilled and deionized water and were routinely degassed and sonicated under vacuum after filtration. Millex disposable filter units (0.22 µm) were purchased from Millipore Corporation (Bedford, MA), and fused-silica capillary columns were obtained from Scientific Glass Engineering (Austin, TX), and Polymicro Technologies (Phoenix, AZ).

Instrumentation

A laboratory-made instrument with fluorescence detection and a commercial instrument with ultraviolet detection were used. The laboratory-made instrument consisted of a capillary electrophoresis section and a fluorescent microscope with a photomultiplier linked to a computer as a detection system (see Figure 2). The microscope was a Nikon Optiphoto II model epiillumination fluorescence unit (Nikon Instrument Group, Garden City, NY), attached to a computerized single-photon detector photomultiplier (Photon Technology International, South Brunswick, NJ). The microscope consisted of the following: **(a)** a 50 W high pressure mercury excitation lamp (model HV 1010 1AF), **(b)** a 380-425 nm band pass filter, **(c)** a 430

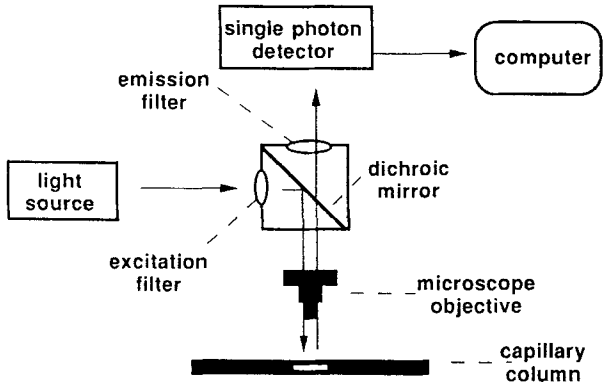

Figure 2. The Capillary Electrophoresis (CE) Instrument. The fluorescence system uses a fluorescent microscope as an amplifier and a single photon detection unit linked to a computer as the fluorescence detector. The excitation lamp is a 50 W high pressure mercury lamp, the band pass filter a 380-425 nm filter, a 430 nm dichroic mirror, and the emission filter is a 450 nm filter. A direct current, high-voltage power supply was used. High-voltage was applied through platinum iridium electrodes for both electrokinetic injections and sample separations. Samples were injected electrokinetically at 25 kV for 45 sec, and analytes are separated for 1 hr at 25 kV. See text for details.

nm dichroic mirror, and **(d)** a 450 nm emission filter (V-2A Nikon filter cube). A direct current, high-voltage power supply was used (Spellman High Voltage Electronics, Plainview, N.Y.). High-voltage was applied through platinum iridium electrodes for both electrokinetic injections and sample separations. Samples were injected electrokinetically at 25 kV for 45 sec and analytes were separated for 1 hr at 25 kV.

Sample preparation

Stock solutions of standard samples were individually prepared by dissolving LHRH (3.2 mg/ml), ßEND (0.5 mg/ml), and NPY (0.5 mg/ml) in 0.1 M sodium tetraborate (borax) buffer, pH 9.0. Luteal and follicular **in vivo** release PPC perfusate samples (obtained as described above) were stored frozen in 100 µl aliquots until derivatized.

Sample derivatization

For CE analysis without fluorescamine derivatization, assay samples were diluted to desired concentrations with sample dilution buffer (0.1 M sodium tetraborate buffer, pH 9.0) and directly transferred to microcentifuge tubes and placed on the turntable of the CE instrument. For CE analysis of fluorescamine derivatives, however, an aliquot of the 100 µl stored solution of the respective analyte samples (concentration ranging from 2.1 to 1250 µg, or from 7.4 to 172.2 nanomole per 100 µl reaction mixture) were transferred to a 500 µl microcentrifuge tube, and their total volume was adjusted to 70 µl by addition of sample dilution buffer. Derivatization was performed by the addition of 30 µl of fluorescamine solution (3 mg/ml fluorescamine in acetone, containing 20 µl pyridine) to the sample while continuously and vigorously vortexing. After approximately 2 min, the content of the microcentrifuge tube was transferred to the turntable of the CE instrument for analysis.

Separation conditions

When using the laboratory-made instrument samples were injected into the capillary column electrokinetically at 25 kV for 45 sec. At the completion of each run, the capillary column was sequentially washed by injection of 2.0 N sodium hydroxide solution, 0.1 N sodium hydroxide solution, distilled-deionized water, and then regenerated with running buffer. The CE separations reported were performed using 0.05 M sodium tetraborate buffer, pH 8.3, containing 0.025 M LiCl. The CE instrument was equipped with a 125 cm (100 cm to the detector) x 75 µm I.D. capillary column, and the separation was performed at 25 kV for 1 hr. Under these conditions, approximately 120 nl (2.7 nl/sec) was injected into the capillary column. The analytes were monitored at a wavelength of 475 nm (emission).

Comparison with a UV detection CE-based assay

For comparative purposes, fluorescamine derivatized standards and PPC samples were also injected into a commercially available CE instrument (P/ACE System 2000, Beckman Instruments, Palo Alto, CA) fitted with a UV detection system. Fluorescamine is a nonfluorescent substance that reacts efficiently with primary amino acids to form intensily fluorescent substances and with secondary amino acids to form nonfluorescent aminoenone type chromophores easily detected at the low UV region [for fluorescence derivatization see 21-24, 29-32]. In the UV system used, the capillary is housed in a cartridge constructed in order to allow a flow of recirculating liquid for Peltier-temperature control of the capillary column. Samples

were stored in a microapplication vessel assembly consisting of a 150 µl conical microvial inserted into a standard 4 ml glass reservoir and held in position for injection by an adjustable spring. To minimize evaporation of the sample volume (100 µl), about 1-2 ml of cool water was added to the microapplication vessel housing the microvial. The external water serves as a cooling bath for the sample in the microvial and as a source of humidity to prevent sample evaporation and concentration. After insertion of the microvial, the microapplication vessel assembly was covered with a rubber injection septum and placed into the sample compartment of the CE instrument. The analysis program was initiated and the first sample automatically injected into the capillary by a positive nitrogen pressure of 0.5 psi (3500 Pa) for 20 sec. The instrument was equipped with a 57 cm (50 cm to the detector) x 75 µm I.D. capillary column, and the separation was performed at 18 kV. Capillary temperature for all experiments was maintained at 25°C during the run. Under these conditions, approximately 120 nl (6 nl/sec) was injected into the capillary column of the Beckman instrument [25]. The analytes were monitored at a wavelength of 214 nm. Peak visualization and data acquisition were performed using the UV detection system of the CE instrument and the System Gold Chromatography Software package (Beckman Instruments, San Ramon, CA). Data integration was also carried out with a model D-2500 Chromato-Integrator (Hitachi Instruments, Inc., Danbury, CT).

RESULTS

Figure 3 shows RIA-derived values for LHRH, ßEND, and NPY for each one of the samples allocated to either a luteal or a follicular pool, which were used as constant sample source for developing the CE-based assay. Please notice that this is the maximum information that can be obtained from a single PPC sample (100 µl/10 min) by RIA, and therefore is the minimum information required from a CE-based system. While LHRH values range from 0.1-1.0 pg/100 µl PPC perfusate, those from ßEND and NPY run roughly from 1-100 pg/100 µl PPC perfusate, with the exception of a few hours in the follicular phase when ßEND reaches very high values. Therefore, LHRH is the limiting peptide since it is found at the lowest concentration.

Figure 4 shows electropherograms of fluorescamine-derivatized neuropeptides as determined by fluorescence detection at 475 nm emission. The bottom panel shows the fluorescamine derivatized neuropeptide standards and the upper panel shows a representative electropherogram of a fluorescamine derivatized neuropeptides

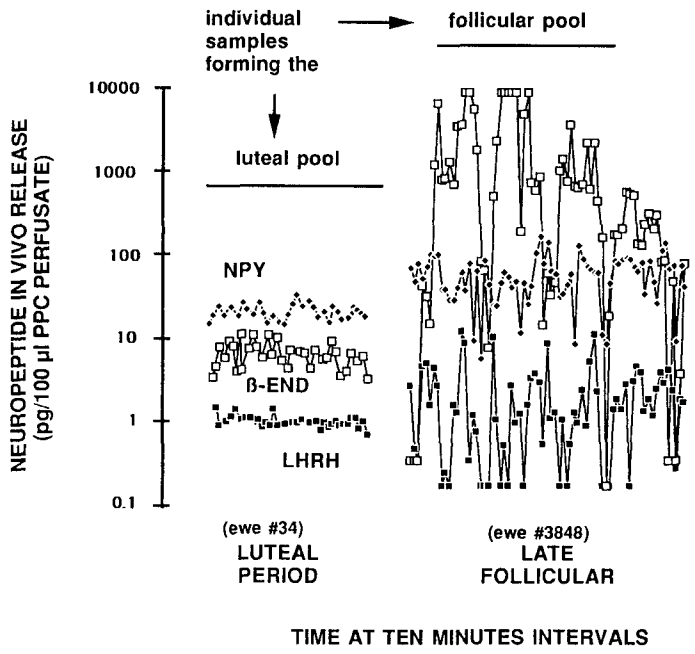

Figure 3. Radioimmunoassay-Based Content of Each Individual Sample Forming the Pool. Samples of pulsatile **in vivo** neuropeptide release were obtained at 10 min intervals, during both the luteal and the follicular phase of the ewe estrous cycle, for 5 hr (luteal period) to 10 hr (follicular phase).

found in a PPC sample (100 µl follicular pool sample). In both profiles peak 1 is LHRH, peak 2 is NPY, and peak 3 is ßEND. The electropherograms of NPY and ßEND standards are not resolved as single peaks, and they probably represent either variants of each neuropeptide or impurity products. It is worth noticing that the quality control information for these neuropeptides provided by the manufacturer indicates the presence of a single peak for each one, as shown by HPLC and UV detection at 214 nm, a less sensitive system than the one we used.

To optimize the degree of derivatization of the analytes with fluorescamine under the reaction conditions used, i.e., pH 9.0 in a sodium tetraborate buffer at 25°C, a fixed amount of LHRH (115

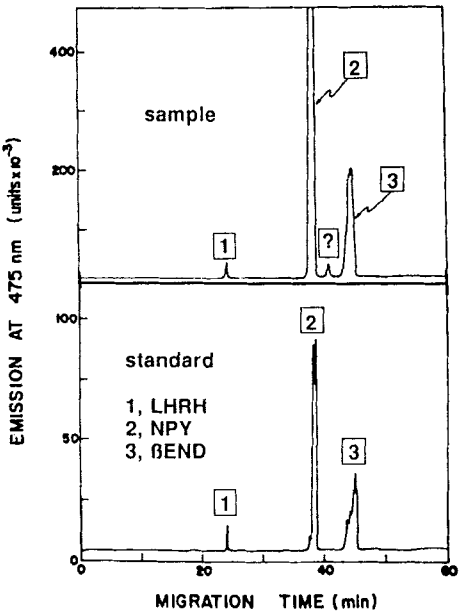

Figure 4. Capillary Electrophoresis Profile of Fluorescamine-Derivatized Neuropeptides Analyzed by Fluorescence Detection. The profile at the bottom represents a mix of neuropeptide standards analyzed by fluorescence detection: Peak 1 is luteinizing hormone-releasing hormone (LHRH); peak 2 is neuropeptide Y (NPY); and peak 3 is ß-endorphin (ßEND). The electropherogram on top is that of a follicular sample. All three neuropeptides were detected.

nmole) was reacted with increasing concentrations of the fluorescamine reagent. Peak area of the derivative appeared to be maximal at a fluorescamine concentration of approximately 300 nmole (per 100 µl reaction mixture volume). Therefore, for this neuropeptide, a two to three fold molar excess of fluorescamine reagent should be sufficient to saturate the reaction mixture and form an optimal fluorescamine-LHRH derivative (results not shown). Thus, using these experimental conditions, we assessed the linearity of the derivative peak area as a function of LHRH concentration (see below).

Figure 5 shows the electropherograms of a control fluorescamine solution and of a fluorescamine-derivatized mixture of

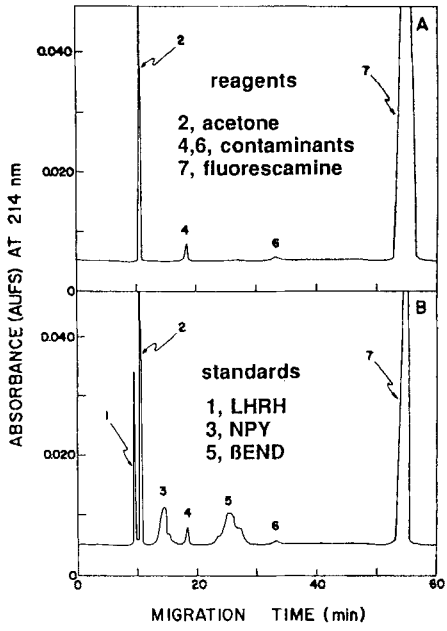

Figure 5. Capillary Electrophoresis Profile of Fluorescamine-Derivatized Neuropeptide Standards Analyzed by UV Detection. **(A)** Electropherogram of the fluorescamine solution control. Peak 2 represents acetone; peaks 4 and 6, reagent contaminants; and peak 7, fluorescamine reagent. **(B)** Electropherogram of LHRH, NPY, and ßEND. Peak 1 represents LHRH; peak 2, acetone; peak 3, NPY; peak 4, reagent contaminants; peak 5, ßEND; peak 6, reagent contaminant; and peak 7, fluorescamine reagent.

LHRH, NPY, and ßEND standards, as determined by UV detection at 214 nm. Figure 5A shows a fluorescamine reagent control run. Peak 2 represents the comigration of the organic solvents acetone and pyridine, peaks 4 and 6 are reagent contaminants, and peak 7 is the fluorescamine reagent. Figure 5B shows that all three fluorescamine-derivatized neuropeptides, LHRH (peak 1), NPY (peak 3), and ßEND (peak 5), are well separated from each other and from the peaks corresponding to the constituents of the derivatization reagent, fluorescamine (peak 7) and the organic solvents acetone and pyridine (comigrating at peak 1). The observed decreased peak area

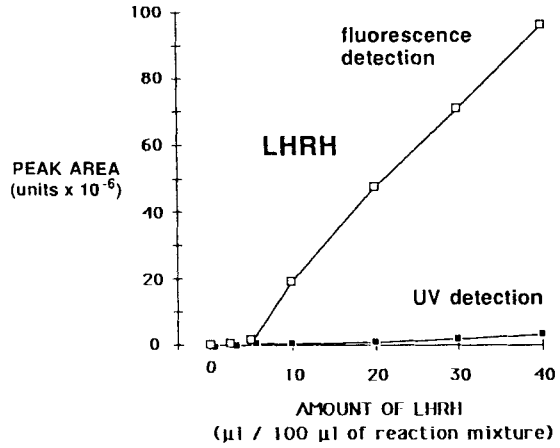

Figure 6. Relationship Between Peak Area of the Derivatized Analyte Form and the Concentration of Reacting LHRH. The linearity of the formation of fluorescamine-derivatized neuropeptide was calculated by reacting increasing concentrations of LHRH (ranging from 6.7 nanomoles to 108 nanomoles/100 µl reaction mixture) with an excess amount of fluorescamine reagent (324 nanomoles/100 µl reaction mixture). The procedure was carried out as described in Material and Methods. The separation buffer consisted of 0.05 M sodium tetraborate buffer, pH 8.3, containing 0.025 M LiCl.

for the fluorescamine (Figure 5B, peak 7), in comparison with the control value (Figure 5A, peak 7), confirms that a significant amount of the reagent is immediately consumed in reaction with the analytes. No LHRH, NPY, or ßEND peaks were detected using UV detection in follicular pool samples, at the injected volumes (120 nl of a 100 µl reaction mixture, having 40 µl of original PPC perfusate sample). In contrast, fluorescence detection clearly allowed the visualization of these neuropeptides in these samples at the same injected volumes and conditions (see Figure 4).

Figure 6 and Table 1 show that a linear response is observed at LHRH concentrations ranging from 8 µg (6.7 nanomoles) to 128 µg (108 nanomoles) of reaction mixture. Table 1 shows a typical concentration of LHRH standard in 100 µl reaction mixture or in 120 nanoliters injected into the CE capillary column and assessed by ei-

TABLE 1. Analysis of a Fluorescamine-Derivatized LHRH Standard Solution Using Capillary Electrophoresis and Ultraviolet (UV) or Fluorescence (FLU) Detection

Amount of LHRH Present in 100 µl Reaction Mixture				Amount of LHRH Injected into the Capillary Column			
Detection	µl	µg	nanomole	nL	ng	picomole	peak area (units x 10^{-6})
UV	2.5	8	6.7	120	9.6	8.1	0.10
	5	16	13.5	120	19.2	16.2	0.30
	10	32	27.0	120	38.4	32.4	0.50
	20	64	54.0	120	76.8	64.8	1.20
	30	96	81.0	120	115.2	97.2	2.10
	40	128	108.0	120	153.6	129.6	3.20
FLU	2.5	8	6.7	120	9.6	8.1	0.03
	5	16	13.5	120	19.2	16.2	0.80
	10	32	27.0	120	38.4	32.4	19.40
	20	64	54.0	120	76.8	64.8	46.60
	30	96	81.0	120	115.2	97.2	71.00
	40	128	108.0	120	153.6	129.6	96.10

For this experiment, increasing amounts of LHRH were reacted with a fixed concentration of fluorescamine reagent (324 nmole/100 µl reaction mixture). The separation buffer consisted of 0.05 M sodium tetraborate buffer, pH 8.3, containing 0.025 M LiCl. The stock solution of LHRH (MW 1182) was 3.2 mg/ml.

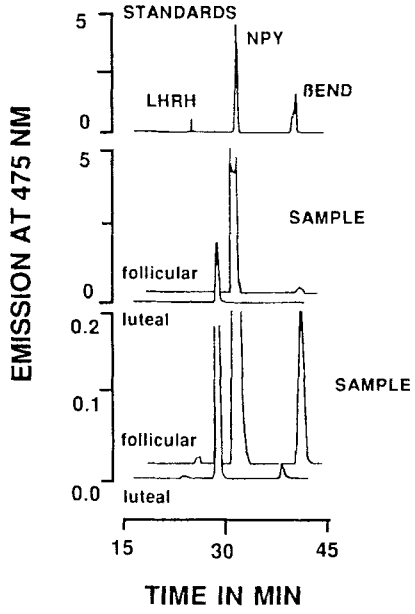

Figure 7. Electropherogram of a Luteal and a Follicular Pool Aliquot. The electropherogram at the top panel shows the LHRH, NPY, and βEND standards. The middle and bottom panel show the results of both a luteal and a follicular sample. The scale in the electropherogram at the bottom has been expanded to show the low neuropeptide content in luteal samples. The increase in NPY and βEND observed between luteal and follicular ewes was clearly distinct. However, the sensitivity of the assay is only borderline for LHRH, the neuropeptide found at the lowest concentration in a single PPC sample obtained at 10 min intervals.

ther UV or fluorescence detection. Detection sensitivity is considerably increased when using fluorescence detection. For example, when using 30 µl of standard/100 µl of reaction mixture (96 µg of LHRH) or 115 ng (97.2 picomoles) in 120 nanoliters of volume injected into the column, UV detection was 2 x 10^{-6} units and fluorescence detection was 71 x 10^{-6} units. That is a 34-fold increase in peak area.

Figure 7 shows the electrophoretical profile of the mid-luteal and the follicular in vivo neuropeptide release pools. The electro-

pherogram at the top shows the LHRH, NPY, and ßEND standards. The middle and bottom panel show the results of both the luteal and the follicular pool. The scale in the electropherogram at the bottom has been expanded to show better the low neuropeptide content in the luteal pool. The increase in NPY and ßEND observed between luteal and follicular ewes was clearly distinct. However, the sensitivity of the assay is only borderline for LHRH, the neuropeptide found at the lowest concentration in PPC sample obtained at 10-min intervals (100 µl/10 min).

DISCUSSION

Rapid progress in the determination of neuropeptides has enabled more stringent conditions for their analysis in tissues and biological fluids. At present, radioimmunoassays (RIAs) are the most common methods used to determine these biological active substances. However, antibodies used in RIAs are targeted to determine one specific neuropeptide at a time. Thus, sample volume might become a limiting issue when multiple neuropeptides must be assessed in the same sample by RIAs, especially when their sample concentration is low. Other drawbacks of RIAs are the use of radioactive material and the need to have access to high-titre-specific antibodies for each neuropeptide to be measured. In general, RIAs allow the determination of picogram levels of neuropeptides per assay tube. In contrast to RIAs, high performance liquid chromatography (HPLC), another commonly used technique, enables the determination of several neuropeptides simultaneously. Although HPLC is a technique with high separation power, it is only able to detect neuropeptides at several nanograms per injection when coupled to UV detection at 214 nm. This sensitivity is usually unacceptable even to detect tissue level concentrations of these substrates. Furthermore, most reagents used in the fluorescence derivatization of amines have properties that limit their usefulness with peptides, since their reaction systems tend to precipitate in the presence of HPLC solvents [21-24]. Current sampling techniques to assess in vivo neuropeptide release (e.g., push-pull cannula and microdialysis sampling from discrete brain areas) generate perfusate samples with low neuropeptide content (low pg level) and low sample volume (low µl level). Thus, additional manipulations are required to concentrate these **in vivo** release perfusate samples in order to reach the sensitivity level of HPLC assays. An alternative approach to assess multiple neuropeptides from a single **in vivo** perfusate sample is to separate its neuropeptides by HPLC, collect

fractions, and then assaying the fractions by RIAs. However, quantification requires the use of adequate internal standard controls. It is noteworthy to mention that during **in vivo** release of neuropeptides, as well as during preparation of samples extracted from biological fluids and/or tissues, some biochemical processing of neuropeptides (e.g., deamidation) might occur [26]. These changes might yield peptide fragments that RIA can not detect, but that might be related to changes in biological activity of the peptide, as for example a differential role in volume versus wiring transmission [27]. Most peptide fragments are usually resolved by HPLC.

The utility of CE for the analysis of neuropeptides has been previously demonstrated [12-14, 28]. However, because of its limitations in detection sensitivity, it is essential to develop CE conditions to maximize detection, in order to visualize small amounts of neuropeptides and other constituents of brain tissue. One of the strategies to enhance detection is the use of derivatization techniques, as for example, the use of a chromophore to enhance the detection signal either in the UV and/or fluorescence region, thus increasing sensitivity [29, 30]. Fluorescamine is one of these chromophores which reacts efficiently with primary amino acids to form intensely fluorescent substances, and with secondary amino acids to form nonfluorescent aminoenone type chromophores that are easily detected at the low UV region. Conjugation to fluorescamine also enhances the degree of resolution of the neuropeptides, enabling baseline separation of the conjugated analytes. Fluorescamine has been applied by others to peptide fluorescence derivatization in liquid chromatography in the presence of HPLC solvents [24, 31-34]. However, HPLC solvents limit the use of fluorescamine since it tends to precipitate under these conditions. This is not a problem in the CE-based assay described in this report, since its CE separation buffer does not have HPLC solvents.

Several approaches have been developed recently to increase sensitivity of CE systems, in addition to the use of derivatization techniques, as the one described in this and previous reports [14, 29]. Such improvements include: **1)** the use of special lenses [35], for example, concave lenses to concentrate the light beam to the capillary center, thus reducing light scattering and increasing sensitivity; **2)** the development of a multi-refractive system [36], for example, using a silver coating to amplify the light beam within the capillary, thus increasing capillary light output downstream and *therefore increasing* sensitivity; **3)** the use of a Z-shaped capillary [37], for example, by changing the spatial configuration of a portion

of the capillary to increase the path length of the light beam within the capillary, thus increasing sensitivity; **4)** the use of a bundle of capillaries [38] to increase the loading volume into the section of the capillary in contact with the detection system, thereby increasing the total sample volume, thus increasing sensitivity; **5)** the use of an analyte concentrator [39] to increase the loading volume into the section of the capillary in contact with the detection system, thereby saturating an in-line affinity segment of the capillary located at the injection site, from which a specific analyte (e.g., if an antibody is used) or nonspecific analytes (e.g., if a C-18 support matrix is used) are captured and then eluted, thus increasing sensitivity.

The Comité International des Pois et Mesures [40] has recently accepted zeptomole and yoctomole as the smallest detection sensitivity values (1 zeptomole= 10^{-21} moles = 600 molecules; 1 yoctomole = 10^{-24} moles = 0.6 molecules). This is probably the range of significance for the determination of neuropeptides and other neurochemicals found in minute amounts, at neuroendocrine controlling sites. Under the best circumstances, RIAs can reach detection limits of 10^{-9} to 10^{-10} M. Capillary electrophoresis coupled to laser-induced fluorescence detection is reaching levels of detection for concentrations ranging from 10^{-12} to 10^{-15} M. These values are the most sensitive concentrations so far reported in the literature [16], and they have been obtained using a modification of the standard laser-induced fluorescence detection [41]. This exceptional sensitivity level will be required to determine the electrophoretic characteristics of neuropeptides being released in low concentrations from discrete brain sites, as for example LHRH from the median eminence. The CE-based assay outlined in this report is sensitive enough to determine the electrophoretic characteristics of neuropeptides released in large amounts from this site (e.g., NPY and ßEND). Slight structural modifications of the neuropeptides being released (e.g., deamidation) might exist under different physiological (reproductive) conditions [26], thus giving origin to neuropeptide variants, which might affect their diffusion characteristics and therefore play a major role in a volume transmission context [27]. We should also keep in mind that slight modifications in the neuropeptide structure might render it unrecognizable by specific antibodies and therefore by RIA.

In conclusion, we developed a CE-based assay for the simultaneous determination of LHRH, ßEND and NPY in PPC samples from the ewe ME. This assay uses CE for the separation of these neuropeptides and

their derivatization with the fluorogenic chromophore fluorescamine to amplify their signal. The CE-based assay (120 nl of PPC perfusate injected into the system) allows the simultaneous electrophoretic assessment of endogenous NPY and ßEND (found at levels between 10-100 pg/100 µl PPC perfusate/10 min as resolved by RIA). However, simultaneous detection of endogenous LHRH (present at levels between 0.1-1 pg/100 µl PPC perfusate/10 min) might only be achieved when laser-induced fluorescence detection of derivatized neuropeptides is coupled to additional sensitivity enhancers already in existence.

ACKNOWLEDGEMENTS

This work was supported in part by USDA 89-37240-4587 to J.P. Advis.

REFERENCES

1. Kendrick, K.M. (1989). In: **Neuroendocrine Peptide Methodology** (P. Conn, Ed.), pp. 229-252, Academic Press, New York.
2. Kasting, N.W., and J.B. Martin (1989). In: **Neuroendocrine Peptide Methodology**, (P. Conn, Ed.), pp. 253-264, Academic Press, New York.
3. Lunte, C.E., D.O. Scott, and P.T. Kissinger (1991). Anal. Chem., **63:** 773A-780A.
4. Fink, G., R. Rosie, and E. Thomson (1991). In: **Volume Transmission in the Brain**, (K. Fuxe and L. Agnati, Eds.), pp. 195-212, Raven Press, New York.
5. Goodman, R. (1988). In: **The physiology of Reproduction** (E. Knobil and J. Neill, Eds.), pp. 1929-1971, Raven Press, New York.
6. Levine, J., and V. Ramirez (1986). In: **Methods in Enzymology**, (P.M. Conn, Ed.), pp. 466-498, Academic Press, New York.
7. Conover, C.D., R.O. Kuljis, D.K. Sarkar, and J.P. Advis (1990). Soc. Neurosci Abstr., pp. 167.14.
8. Advis, J.P., C.D. Conover, J.K. McDonald, and R.O. Kuljis (1990). Ann. N.Y. Acad Sci., **611:** 468-470.
9. Silverman, A.J. (1988). In: **The Physiology of Reproduction**, (E. Knobil and J. Neill, Eds.), pp. 1283-1304, Raven Press, New York.
10. Conover, C.D., R.O. Kuljis, J. Rabii, and J.P. Advis (1992). Neuroendocrinology, in press.
11. Prasad, B., C.D . Conover, D.K. Sarkar, J. Rabii, and J.P. Advis (1992). Neuroendocrinology, in press.

12. Advis, J.P., L. Hernandez, and N.A. Guzman (1989). Peptide Res., **2:** 389-394.
13. Guzman, N.A., L. Hernandez, and J.P. Advis (1990). In: **Current Research in Protein Chemistry: Techniques, Structure, and Function** (J. Villafranca, Ed.), Chapter 19, pp. 203-216, Academic Press, New York.
14. Guzman, N.A., M.A. Trebilcock, and J.P. Advis (1991). In: **Techniques in Protein Chemistry II** (J.J. Villafranca, Ed.), Chapter 4, pp. 37-54, Academic Press, New York.
15. Wallingford, R.A., and A.G. Ewing (1990). Adv. Chromatogr., **29:** 1-76.
16. Hernandez, L., N. Joshi, J. Escalona, and N.A. Guzman (1991). J. Chromatogr., **559:** 183-196.
17. Advis, J.P., R.O. Kuljis, and G.S. Dey (1985). Endocrinology, **116:** 2410-2418.
18. Kuljis, R.O., and J.P. Advis (1989). Endocrinology, **124:** 1579-1581.
19. Sarkar, D.K., and S. Yen, (1985). Endocrinology, **116:** 2075-2079.
20. McDonald, J.K., J.I. Koenig, D.M. Gibbs, P. Collins, and B.D. Noe (1987). Neuroendocrinology, **46:** 538-541.
21. Lingeman, H., W.J.M. Underberg, A. Takadate, and A. Hulshoff (1985). J. Liq. Chromatogr., **8:** 789-874.
22. Ogden, G., and P. Földi (1987). LC.GC, **5:** 28-40.
23. Lunte, S.M., and O.S. Wong (1989). LC.GC, **7:** 908-916.
24. Newcomb, R. (1992). LC.GC, **10:** 34-39.
25. Harbaugh, J., M. Collette, and H. Schwartz (1990). Beckman Instruments Technical Information Bulletin No. TIBC-103 (4SP-890-10B).
26. Wright, H.T. (1991). Crit. Rev. Mol. Biol., **26:** 1-52.
27. Fuxe, K. and L.F. Agnati (1991). In: **Volume Transmission in Brain** (K. Fuxe and L.F. Agnati, Eds.), pp. 1-9, Raven Press, New York.
28. Guzman, N.A., L. Hernandez, and B.G. Hoebel (1989). BioPharm, **2:** 22-37.
29. Guzman, N.A., J. Moschera, K. Iqbal, and A.W. Malick (1992). J. Liq. Chromatogr., **15:** 1163-1177.
30. Guzman, N.A., J. Moschera, C.A. Bailey, K. Iqbal, and A.W. Malick (1992). J. Chromatogr., **598:** 123-131.
31. Felix, A.M., V. Toome, S. De Bernardo, and M. Weigele (1975). Arch. Biochem. Biophys., **168:** 601-608.
32. Rubenstein, M., S. Chen-Kiang, S. Stein, and S. Udenfriend (1979). Anal. Biochem., **95:** 117-121.
33. Tsuda, T., Y. Kobayashi, A. Hori, T. Matsumoto, and O. Suzuki (1988). J. Chromatogr., **456:** 375-381.

34. Brown, D.S., and D.R. Jenke (1987). J. Chromatogr., **410:** 157-168.
35. Tsuda, T., J.Y. Sweedler, and R.N. Zare (1990). Anal Chem., **62:** 2149-2152.
36. Wang, T., J.H. Aiken, C.W. Huie, and R.A. Hardwick (1991). Anal. Chem., **63:** 1372-1376.
37. Chervet, J.P., R.E.J. vanSoest, and M. Ursem (1991). J. Chromatogr., **543:** 439-449.
38. Guzman, N.A., M.A. Trebilcock, and J.P. Advis (1991). Anal. Chimica Acta, **249:** 247-255.
39. Guzman, N.A., M.A. Trebilcock, and J.P. Advis (1991). J. Liq. Chromatogr., **14:** 997-1015.
40. Chenm, D.Y., H.P. Swedlow, H.R. Harke, J.Z. Zhang, and N.J. Dovichi (1991). J. Chromatogr., 559: 237-246.
41. Hernandez, L., R. Marquina, J. Escalona, and N.A. Guzman (1990). J. Chromatogr., **502:** 247-255.