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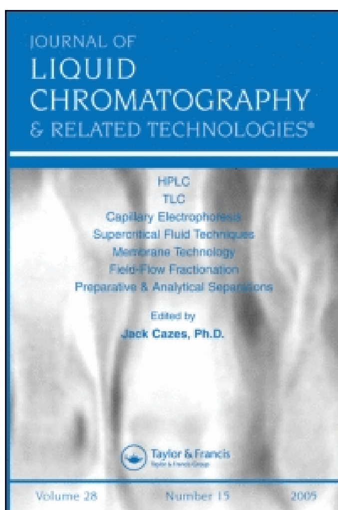

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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To cite this Article Jaffe, Howard , Loeb, Marcia , Hayes, Dora K. and Holston, Nancy(1982) 'Rapid Isolation of Nanogram Amounts of Crustacean Erythrophore Concentrating Hormone from Invertebrate Nerve Tissue by RP-HPLC', Journal of Liquid Chromatography & Related Technologies, 5: 7, 1375 — 1390
To link to this Article: DOI: 10.1080/01483918208067596
URL: http://dx.doi.org/10.1080/01483918208067596

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JOURNAL OF LIQUID CHROMATOGRAPHY, 5(7), 1375-1390 (1982)

RAPID ISOLATION OF NANOGRAM AMOUNTS OF CRUSTACEAN ERYTHROPHORE CONCENTRATING HORMONE FROM INVERTEBRATE NERVE TISSUE BY RP-HPLC *

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ABSTRACT

A reverse phase-high performance liquid chromatography (rp-hplc) method was developed for the rapid isolation of nanogram amounts of crustacean erythrophore concentrating hormone (CECH) from invertebrate nerve tissue. Tissue homogenates from the shrimp, Paleomenetes pugio, were subjected to a multistep work-up to remove proteins and lipids prior to analysis by rp-hplc. Samples were eluted with a concave gradient of 0.1% trifluoroacetic acid (TFA) verses acetonitrile. Detection at 210 and 254 nm combined with the use of highly efficient and end-capped columns permitted the determination of less than 5 ng of CECH. Pure CECH was isolated from the columns by fraction collection followed by lyophilization of the volatile TFA buffer.

INTRODUCTION

Reverse phase-high performance liquid chromatography (rp-hplc) is rapidly becoming the preferred method for the analysis and isolation of underivatized peptides from tissue. Recent improvements in pumps, detectors, and column technology have made it

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possible to detect or isolate nanogram amounts of peptide hormones from tissue samples (1-8).

Four invertebrate peptide hormones, proctolin (Arg-Tyr-Leu-Pro-Thr), locust adipokinetic hormone (LAKH, p-Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2), crustacean erythrophore concentrating hormone (CECH, p-Glu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2) and mulluscan cardioexcitatory neuropeptide (MCEN, Phe-Met-Arg-Phe-NH_2) have been isolated, identified (9-16) and have become available commercially. In all cases isolation of these peptides required the processing of large numbers of invertebrates followed by size exclusion chromatography. For example, in the isolation of proctolin (9) 125 kg of whole cockroaches, Periplaneta americana, were required to yield 180 μg of pure peptide suitable for structural analysis.

Recently, Stone and Mordue (17) suggested the use of reverse phase-hplc to isolate small quantities of insect neuropeptides. In this paper we describe an hplc method for the rapid isolation of nanogram amounts of peptide hormone from invertebrate nerve tissue. The method is illustrated by a description of the techniques used for the isolation of crustacean erythrophore concentrating hormone (CECH) from 32 pairs of eyestalks removed from the shrimp, Paleomenetes pugio. The average weight of an eyestalk is 2 mg.

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MATERIALS AND METHODS

Chemicals.

Triethylamine (Fisher, Hplc Grade)[1] was purified by fractional distillation through a short Vigreaux column. The middle fraction was further purified by passage through a C_{18} Sep-Pak (Waters Associates). Phosphoric acid (Fisher, Hplc Grade) was used without further purification. Triethylammonium phosphate buffer (TEAP) at pH 2.20 was prepared by the method of Rivier (18). Trifluoroacetic acid (TFA, Baker, Analyzed Reagent Grade) was purified by fractional distillation through a short Vigreaux column and the middle fraction was taken. Hplc grade water was obtained from a Milli-Q System (Millipore). Acetonitrile (Burdick & Jackson or Fisher, Hplc Grade),

ethyl acetate (Fisher, Hplc Grade), methanol (Burdick & Jackson and acetic acid (Baker, Ultrex Grade) were used without further purification. Peptides (Peninsula or Sigma) were stored in the freezer. Standard peptide solutions were made up at 0.50 ± .04 mg in 50 ml 0.1% TFA and stored in the refrigerator.

Crustaceans.

P. pugio (Gulf Specimen Co) were kept in salt water aquaria. Live female lobsters, *Homarus americanus*, were purchased from a local distributor and used immediately.

Hplc.

The hplc system consisted of a gradient liquid chromatograph (Waters Associates) equipped with a Model 660 solvent programmer, Model U6K injector, two Model 6000A pumps, Model 440 absorbance detector and Model 450 variable wavelength detector. The latter was connected to a Model MM 700 memory module (Schoeffel) to provide baseline correction when necessary during the course of the gradient at low wavelengths.

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The columns, Supelcosil LC-18DB (Supelco) or Zorbax C-8 (Dupont) were protected by means of a short guard column of Pelliguard LC-18 (Supelco). Aqueous buffer was filtered and degassed through a type HA filter (Millipore); acetonitrile solutions through a type HF filter. The aqueous buffer was passed through a 61 x 0.78 cm column of Bondapak C_{18}/Porasil B (Waters Associates) installed in line between the high pressure output of the aqueous pump and the mixer (Figure 1).

The columns were equilibrated by running several cycles of the gradient over 1 min followed by isocratic elution at the starting conditions until a stable baseline was achieved. Samples were applied to the equilibrated columns which were then eluted with a concave gradient (Curve 5) of 90——> 40% aqueous buffer. Buffer systems and optimal hplc conditions as determined experimentally are described in Table 1. The elution was monitored simultaneously at 254 nm (0.01 AUFS) and 210 or 195 nm (0.1 AUFS).

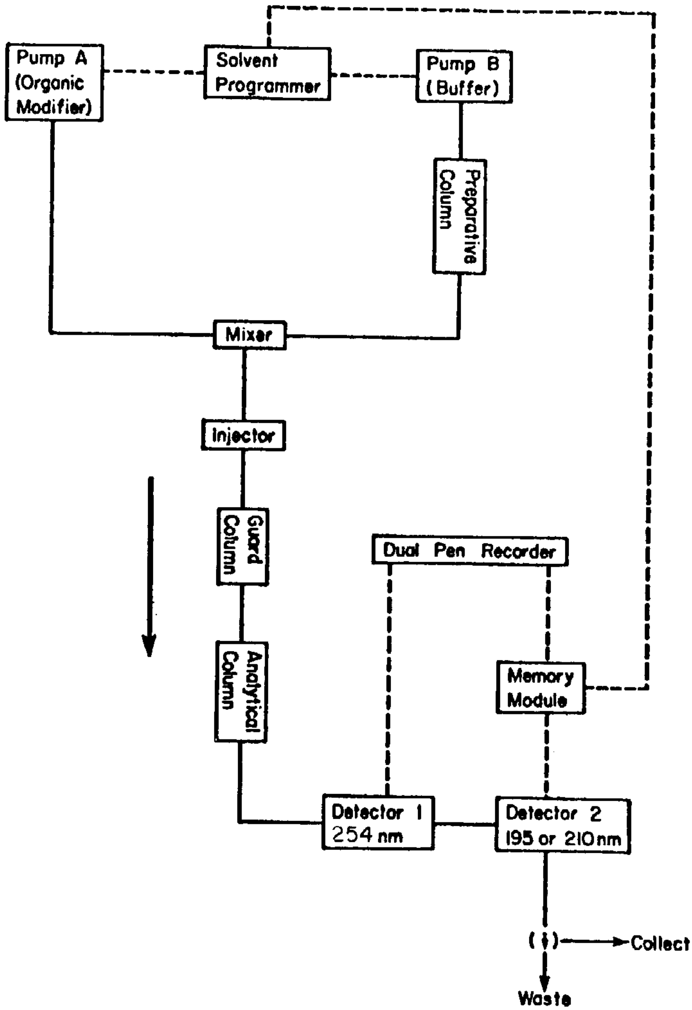

FIGURE 1. Schematic diagram of hplc system. Dashed lines are electrical connections.

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TABLE 1
Buffer Systems and Hplc Conditions Used for Analysis and Isolation of Invertebrate Neuropeptides

Aqueous Phase B	Organic Modifier A	UV Detection (nm)	Hplc Conditions
0.25N TEAP pH 2.20	Acetonitrile	254,195	90——> 40% B/1.0 h/ 1.1 ml/min (curve 5)
0.1% TFA	0.1% TFA in Acetonitrile	254,210	90——> 40% B/1.0 h/ 1.0 ml/min (curve 5)

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Sample preparation.

Pairs of eyestalks from as few as 10 shrimp, *P. pugio*, or eyestalks and brain from the lobster, *H. americanus*, were removed and immediately placed in 1 ml of chilled methanol-water-acetic acid (90:9:1 by vol) in a 1.5 ml polyethylene centrifuge tube (Sarstedt). The tissue was homogenized with a Polytron homogenizer (Brinkmann) equipped with a PT 7 micro probe generator. The homogenate was centrifuged in a Model RC-3B refrigerated centrifuge (Sorvall) at 4000 rpm at 5°C for 30 min. The supernatant was transferred into a clean 1.5 ml polyethylene centrifuge tube and concentrated to minimal volume under a stream of nitrogen. The resulting sample was taken up in 1 ml of 0.1% TFA and extracted with ethyl acetate (3x). The ethyl acetate layer was removed by decantation. Residual ethyl acetate was removed under a stream of nitrogen. Final filtration was accomplished by use of a centrifugal filtration apparatus (Rainin) equipped with a 0.45 Nylon-66 membrane filter centrifuged at 1500 rpm for 10 min at 5°C.

Peptide isolation.

The entire filtered sample (<1 ml) was taken up in 1 cc dispensable plastic syringe (Becton-Dickenson) equipped with a N725 stainless steel needle (Hamilton) and injected into the hplc apparatus. The gradient was begun immediately upon injection. Fractions were collected in polyethylene vials and lyophilized in

a Model 25SRC freeze dryer (Virtis) equipped with a soda trap and outside venting of the vacuum pump. The resulting freeze dried samples were taken up in 100 or 200 μl of 2.7% NaCl for bioassay.

Bioassay.

Peptides and fractions were assayed for their erythrophore concentrating activity as previously described (19) by injecting them into the shrimp, *P. pugio*; induced erythrophore contractions were then assigned values according to the scale of Hogben and Slome (20). Effects of standard solutions of synthetic CECH in 2.7% NaCl on the shrimp erythophores were measured at various times following the injection. Injections (10 μl) were made into shrimp whose eyestalks had been removed 24 hr earlier.

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RESULTS AND DISCUSSION

Many buffer systems have been proposed for reverse phase-hplc of peptides, but in our experience the most versatile for the analysis of a wide range of peptides and small proteins is triethylammonium phosphate (TEAP) vs acetonitrile. This buffer system, first described by Rivier (18), is characterized by its UV transparency down to 190 nm and its excellent resolution. We have recently reported (21) on the use of this buffer system at pH 2.20 in a concave gradient of 90——> 40% TEAP combined with a highly efficient, end-capped column (Supelcosil LC-18DB) to analyze small synthetic invertebrate peptides as well as larger peptides such as insulin (Figure 2). Simultaneous detection at 254 and 195 nm permitted peak ratioing and detection of less than 5 ng of peptide. The TEAP buffer system is also characterized by its excellent recovery of peptides. Peptide peaks collected from the columns could be reinjected after removal of acetonitrile with little loss of peak height. (Table 2). However, despite the fact that TEAP buffer has been reported (18) to be compatible with most biological systems both *in vitro* and *in vivo*, we have found that 0.25N TEAP at pH 2.20 interfered with the bioassay of CECH and LAKH (13). Since TEAP was non-volatile, high salt concentrations

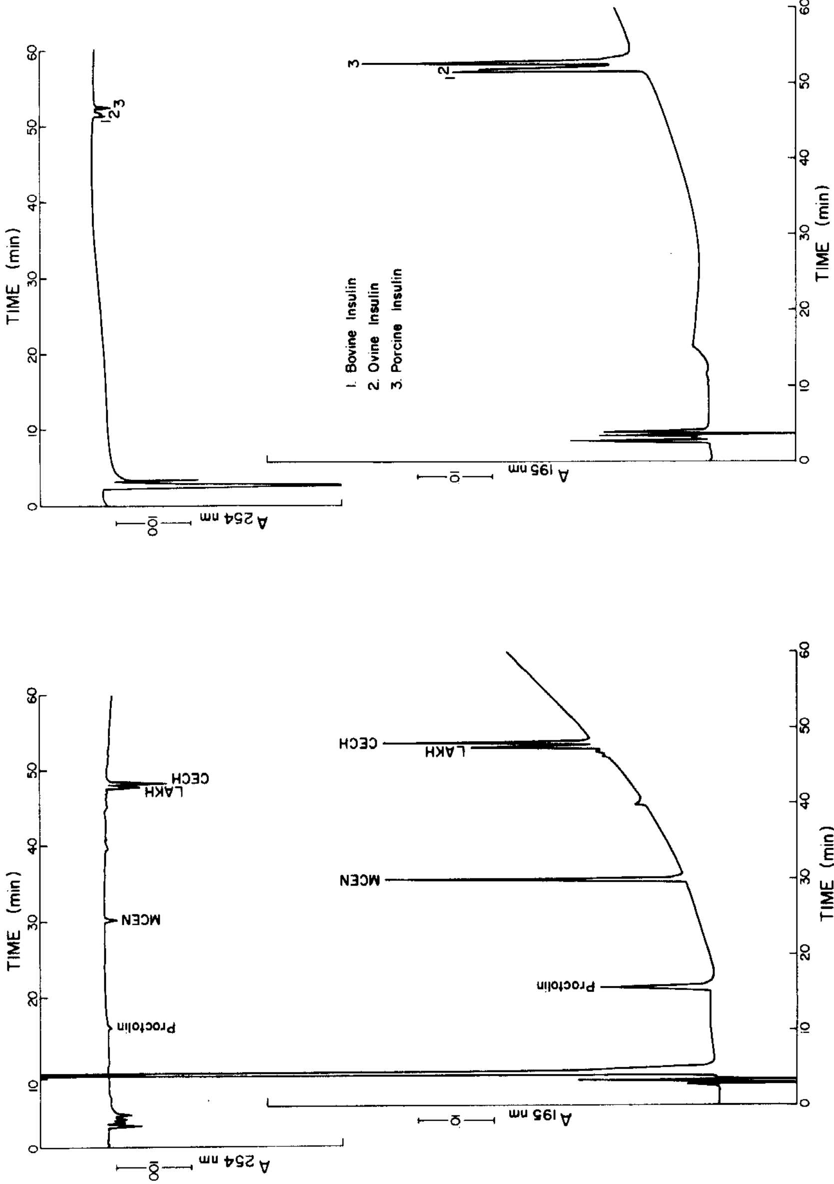

FIGURE 2. Analysis of four invertebrate neuropeptides (left hand trace) and insulins (right hand trace) with TEAP buffer on the Supelcosil LC-18DB column. Buffer system and hplc conditions are described in Table 1.

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TABLE 2
Recovery of Peptides from the Supelcosil LC-18OB Column Using TEAP and TFA Buffers[a]

Peptide	% Recovery TEAP[b]	% Recovery TFA[c]
Proctolin	71	90
leu-enkephalin	61	70
met-enkephalin	94	90
LAKH	-	81
CECH	-	66

a. Buffer and hplc conditions are described in Table 1.
b. Sample reinjected after removal of acetonitrile under nitrogen.
c. Sample reinjected in 0.1 ml 0.1% TFA after lyophilization.

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would be expected when hplc peptide fractions were concentrated prior to bioassay. TEAP-obtained fractions proved to be toxic to bioassay animals and therefore undesirable for bioassay purposes.

A buffer system which eliminates these problems and allows us to lyophilize fractions containing nanogram amounts of peptides for later uptake in physiologically compatible solutions is 0.1% TFA vs acetonitrile (2, 22, 23); it is volatile and yet, like TEAP, is characterized by excellent resolution of small synthetic invertebrate peptides as well as larger peptides such as insulin (Figure 3). Recovery of lyophilized peptide fractions collected from the columns is excellent (Table 2). A disadvantage of the TFA buffer is its UV absorption at low wavelengths. Nevertheless, 0.1% TFA buffer can be run as low as 210 nm without excessive baseline drift by addition of 0.1% TFA to the acetonitrile, so that the TFA concentration does not change during the course of the gradient. However, the absorbance of CECH in 0.1% TFA at 210 nm is about 50% less than in 0.25N TEAP (pH 2.20) at 195 nm (Figure 4).

We have utilized the 0.1% TFA buffer as a basis for development of a method for the rapid isolation of nanogram amounts of neuropeptides from small amounts of invertebrate tissue. The

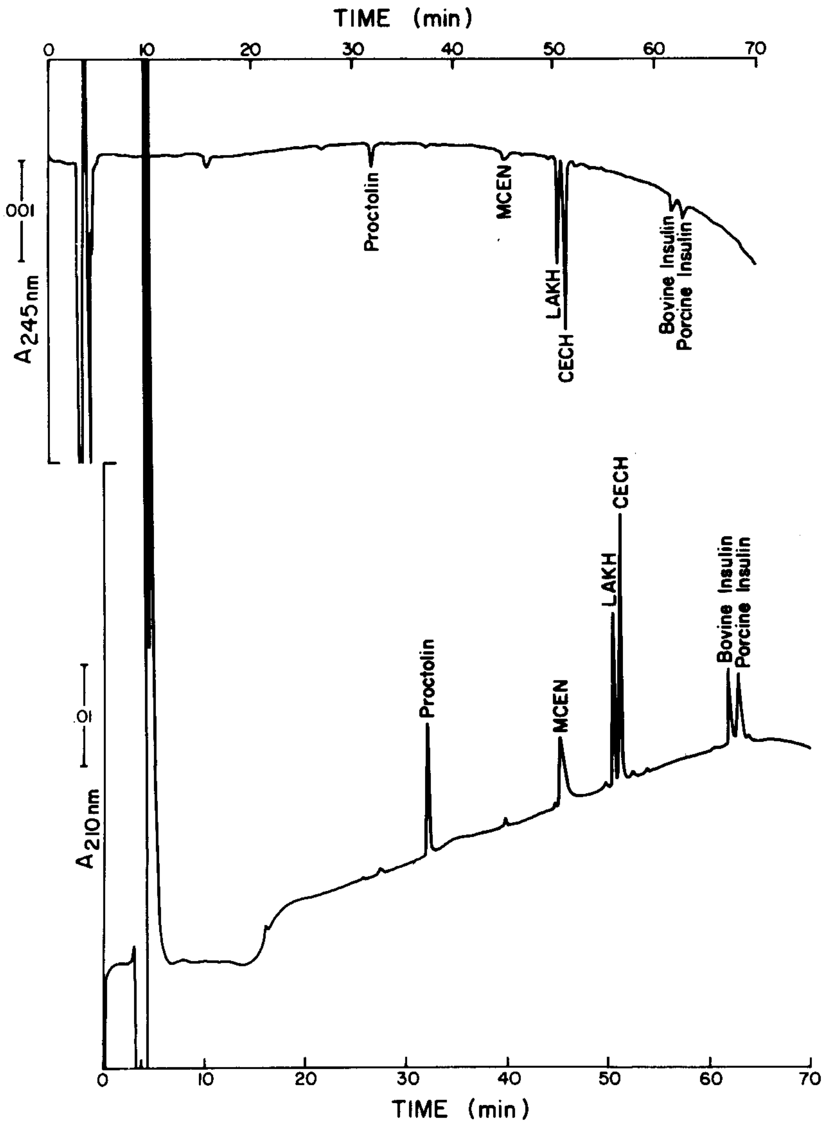

FIGURE 3. Analysis of four invertebrate neuropeptides and insulins with TFA buffer on the Supelcosil LC-18DB column. Buffer system and hplc conditions are described in Table 1.

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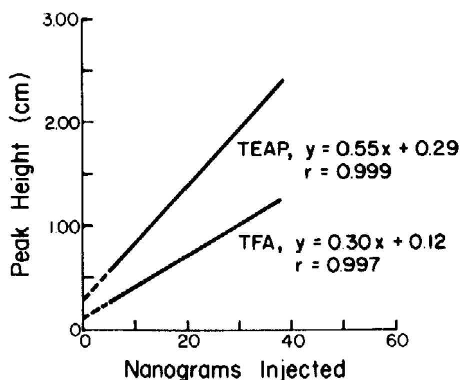

FIGURE 4. Regression analysis of data resulting from plotting peak height (cm) versus ng CECH injected over the range of 4.8-38.4 ng on the Supelcosil LC-18DB column with TEAP and TFA buffer systems. Buffer systems and hplc conditions are described in Table 1.

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method, which involves several preliminary clean-up steps followed by reverse phase-hplc, is illustrated by the isolation of CECH from eyestalks of P. pugio. Eyestalks were homogenized in chilled acidic methanol to precipitate protein. The solvent system employed for this step, methanol-water-acetic acid (90:9:1 by vol) has previously been used by Holman and Cook (24) to homogenize insect tissue in the isolation of proctolin. Methanol was removed, lipids were extracted, and the sample was injected into the instrument with a plastic syringe after a final centrifugal filtration. Each step was designed to minimize losses. Therefore inert plastic was used throughout because CECH, like many other peptides, has been reported to adhere to glass (19). In addition, strongly acidic conditions were employed throughout to inhibit the action of any peptidases present.

Figure 5 is a representative elution profile for the isolation of CECH from the eyestalks of 32 P. pugio. The small sharp peak eluting at 51.5 min was identified as CECH by its retention time, absorbance peak ratio of 210 nm/254 nm, and bioassay of the collected peak. The CECH peak was readily observed with samples derived

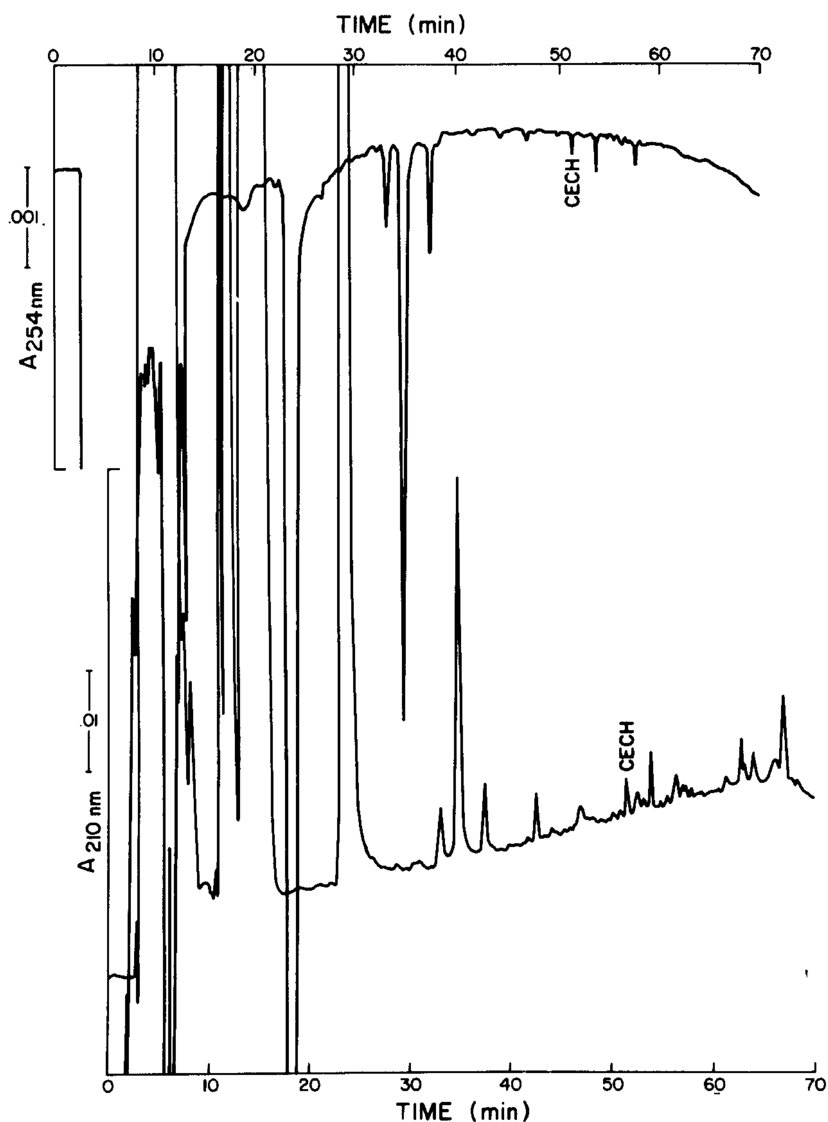

FIGURE 5. Elution profile for the isolation of CECH from the eyestalks of 32 P. pugio on the Supelcosil LC-18DB column with TFA buffer. Buffer system and hplc conditions are described in Table 1.

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TABLE 3
Absorbance Ratio for Invertebrate Neuropeptides on the Supelcosil LC-18DB-TFA and Zorbax C-8-TEAP Systems

Peptide	Absorbance Ratio[a]	
	Supelcosil LC-18DB -TFA[b]	Zorbax C-8-TEAP[b]
Proctolin	5.13	25.14
MCEN	5.86	21.90
LAKH	1.63	3.82
CECH (synthetic)	1.62	3.46
CECH (natural)	1.62	3.46

a. $A_{210\ nm}/A_{254\ nm}$ for TFA, $A_{195\ nm}/A_{254\ nm}$ for TEAP, $A_{210\ nm}$ and $A_{195\ nm}$ at 0.1 AUFS, $A_{254\ nm}$ at 0.01 AUFS.

b Buffer and hplc conditions are described in Table 1.

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from as few as 10 pairs of eyestalks (20 mg). Homogeneity of the peak at 51.5 min was confirmed by analysis on another hplc system. Thus the peak at 51.5 min was collected and analyzed with TEAP buffer on a Zorbax C-8 column, after removal of acetonitrile. This column and buffer system has been previously reported by us (21) to give excellent resolution and sensitivity with invertebrate neuropeptides. The resulting peak on the Zorbax C-8 column at 49.9 min was sharp and had a retention time and absorbance peak ratio (Table 3) consistent with a pure sample of CECH.

The simultaneous monitoring at two wavelengths, 254 and 195 nm (for TEAP) or 210 nm (for TFA) enables the measurement of absorbance peak ratios. The calculation of these ratios has been described (25) as perhaps the most useful of all spectroscopic procedures in liquid chromatography. Absorbance peak ratios for the invertebrate neuropeptides on the Zorbax C-8 and Supelcosil LC-18DB columns for the TFA and TEAP buffers are listed in Table 3.

Retention times of the invertebrate neuropeptides proctolin, MCEN, LAKH, AND CECH were repeatable within 1 min over the course of this study. However, as expected, there was some deterioration of the columns with use as evidenced by peak broadening.

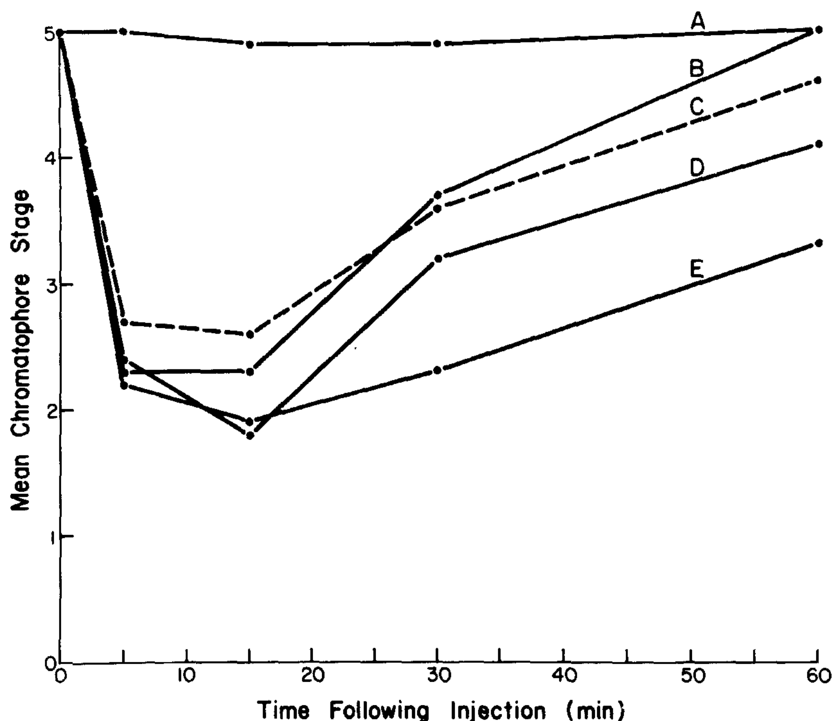

FIGURE 6. Effects of CECH on the erythrophores of P. pugio. Each point represents the mean response of at least 5 animals to 10 μl injections of the following samples: A. Control (2.7% NaCl), B. 0.11 pmol synthetic CECH, C. Natural CECH (1/20 of the collected CECH peak in Fig. 5 after lyophilization), D. 0.55 pmol synthetic CECH and E. 2.20 pmol synthetic CECH.

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As shown in Figure 6, the bioassay for erythrophore concentrating activity in P. pugio detects as little as 0.1 pmol of CECH. The peak at 51.5 min shown in Figure 5 was determined by our standard load response curve (Figure 4) to correspond to 28 ng of CECH. Bioassay of this collected peak after lypholization was positive with 1/20 of this material giving an unequivocal positive response (Figure 6).

In order to test the recovery of peptides in a biological sample, known quantities of commercially available peptides were

TABLE 4
Recovery of Synthetic Invertebrate Neuropeptides by Hplc from Lobster Nerve Tissue Homogenates

Peptide[a]	% Recovery[b]
Proctolin	59
LAKH	74
CECH	78

a. Lobster homogenates were spiked with 1.0 μg of each peptide and worked-up by the method described above.
b. Spiked samples were analyzed on the Supelcosil LC-18DB column with TFA buffer as described above.

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added to brain and eyestalk tissue of the lobster, H. americanus, and the samples processed by our method. Recoveries shown in Table 4 were 59-78%. As an additional test of our methods, lobster brain tissue samples were processed by our method and a fraction collected between 50-60 min was examined for CECH activity by bioassay. Results were positive indicating the presence of CECH-like activity in that fraction. No attempt was made to further isolate or purify any hormone from the fraction.

We believe our hplc method of isolation of CECH from shrimp eyestalks to be a distinct improvement over previously described methods of isolation of the hormone in that we start with much smaller amounts of tissue and utilize only one highly efficient chromatographic separation to yield hplc-pure material.
By contrast, isolation of CECH from eyestalks of Pandalus borealis by Fernlund and Josefsson required 100 g of freeze-dried eyestalks and four time consuming Sephadex separations to yield 20 μg of pure hormone (26).

Our method, illustrated by the isolation of CECH, should be adaptable to the identification of a wide range of peptides from invertebrate nerve tissue. In the case where hplc separation is insufficient to resolve the desired peptide from closely eluting peaks, two consecutive hplc separations on different systems could be employed. The first could utilize the non-volatile TEAP buffer,

followed by the TFA buffer from which isolation of the peptide by lyophilization would be possible. The same or different columns could be employed for the hplc separations.

In summary, a technique is described which allows the rapid and routine identification and purification of nanogram amounts of peptide hormones from mg amounts of invertebrate tissue. The method is based on the superior separation power of hplc, is fast, simple, and should be adaptable to a wide range of peptide hormones.

ACKNOWLEDGMENT

We would like to thank M. E. Getz, P. A. Giang, M. E. Kassouny, J. C. Smith, and C. W. Woods for their critical comments in reading the manuscript, and K. W. Young for his efforts in operating the hplc equipment.

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