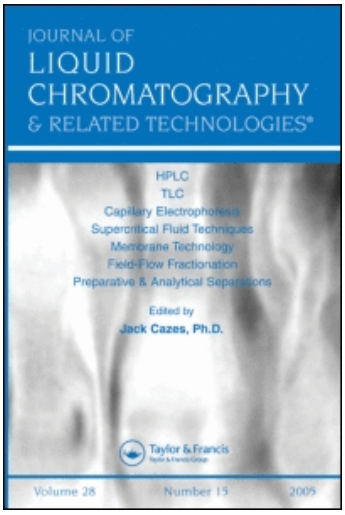

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ANALYSIS OF INVERTEBRATE NEUROPEPTIDES BY RP-HPLC

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ABSTRACT

The application of reverse phase-high performance liquid chromatography to the analysis of four synthetic invertebrate neuropeptides is described. Proctolin (Arg-Try-Leu-Pro-Thr), locust adipokinetic hormone (p-Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2), crustacean erythrophore concentrating hormone (p-Glu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2) and mulluscan cardioexcitatory neuropeptide (Phe-Met-Arg-Phe-NH_2) were analyzed on several different reverse phase columns by means of gradient elution with 0.01M KH_2PO_4, 0.1% H_3PO_4, 0.25N triethylammonium phosphate (TEAP), pH 2.20, or 0.1% trifluoroacetic acid (TFA) versus acetonitrile. Column effluents were monitored at both 254 and 195 nm except in the case of TFA where 254 and 210 nm were monitored. At the lower wavelength computerized background correction was sometimes necessary to correct excessive baseline drift during the course of the gradient run. Best results were obtained on the Supelcosil LC-18DB column with a concave gradient of 90——> 40%B over 1 hr at 1.1 ml/min where B=0.25N TEAP, pH 2.20, A=acetonitrile. With this system less than 5 ng of peptide was detectable. The use of the volatile TFA buffer permitted recovery of peptides from the column effluent by lyophilization.

INTRODUCTION

In recent years reverse phase-high performance liquid chromatography (hplc) has become widely used for the analysis of peptides.

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Recent improvements in hplc hardware and columns combined with the use of ion-pairing buffer systems has made possible the analysis of nanogram amounts of underivatized peptides (1-7).

Four coelamate invertebrate neuropeptides, 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 (8-15) and become available commercially. To our knowledge, hplc analysis has been reported only for LAKH and proctolin. Broomfield and Hardy (16) performed a preparative separation of synthetic LAKH epimers by isocratic elution with methanol-water on a μ-Bondapak[1] C_{18} column. Holman and Cook (17) reported the preparative separation of proctolin on Poragel PN and the hplc analysis of PTC-derivatives of proctolin and several synthetic analogues on methanol-deactivated μ-Porisil. The minimum amount of proctolin that could be quantitatively measured by this method was 25-30 ng. The hplc analysis of underivatized proctolin and several synthetic analogues was reported by Starratt and Stevens (18). Several ion-pairing buffer systems were run isocratically on the μ-Bondapak fatty acid analysis and μ-Bondapak C_{18} columns. Good separations were obtained but sensitivity was limited because the U.V. absorption of the buffers required monitoring at 225 nm. Recently, preparative separations of proctolin on μ-Bondapak C_{18} with the volatile buffer ammonium acetate versus acetonitrile were reported by O'Shea and Adams (19). Good separations were apparently obtained but, as before, sensitivity was limited by the U.V. absorption of the buffer.

In this paper we report the analysis of the four invertebrate neuropeptides on different reverse phase columns by gradient elution with four ion-pairing buffer systems. Optimum combinations of column and buffer permitted the determination of less than 5 ng of peptide.

MATERIALS AND METHODS

Chemicals.

Triethylamine (Fisher, hplc grade) 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 (20). Trifluoroacetic acid (Baker, analyzed reagent grade) was purified by fractional distillation through a short Vigreaux column. The middle fraction was taken. Potassium dihydrogen phosphate (Baker, ultrex grade) and acetonitrile (Burdick and Jackson or Fisher, hplc grade) were used without further purification. Hplc grade water was obtained from a Milli-Q System (Millipore). Peptides (Peninsula) were made up at 0.50 ± .04 mg in 50 ml 0.1% trifluoroacetic acid (TFA) and stored in the refrigerator. Bovine, ovine, and porcine insulin (Sigma) were made up at 1.0 ± 0.1 mg in 50 ml TEAP buffer.

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) so as to provide baseline correction, when necessary, during the course of the gradient at low wavelengths. Reverse phase columns used are shown in Table 1. Stainless steel columns were protected by means of a short column of Pelliguard LC-18 (Supelco). Aqueous buffers were filtered and degassed through a type HA filter (Millipore) and acetonitrile solutions through a type HF filter. All aqueous buffers were during the course of the run continuously 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). Peptides (25 µl or

TABLE 1
Reverse Phase Hplc Columns Used for Analysis of Invertebrate Neuropeptides

Column	Manufacturer	Guard Col	Particle Size (μ)
Supelcosil LC-18DB	Supelco	Pelliguard LC-18	5
Zorbax TMS	Dupont	Pelliguard LC-18	~6
Zorbax C-8	Dupont	Pelliguard LC-18	~6
Radial-PAK C_{18}, 8 mm ID	Waters Associates	None	10
Radial-PAK C_{18}, 5 mm ID	Waters Associates	None	10

250 ng) in 0.1% TFA or insulins (10 μl or 200 ng) in TEAP buffer were applied to equilibrated columns which were then eluted with a concave gradient (curve 5) of 90⟶ 40% aqueous buffer. Buffer systems and optional hplc conditions as determined experimentally are described in Table 2. The elution was monitored simultaneously at 254 nm (0.01 AUFS) and 195 or 210 nm (0.1 AUFS).

RESULTS AND DISCUSSION

The buffer systems employed in this study have previously been used for the analysis of mammalian peptides (20-23). The TEAP, H_3PO_4 and KH_2PO_4 buffers are non-volatile and were found by us to be usable at 195 nm. The TFA buffer is volatile but could be used only as low as 210 nm because of its absorbance at lower wavelengths.

The columns used are of two types. The Supelcosil LC-18DB, Zorbax C-8 and Zorbax TMS are conventional stainless steel columns containing microparticulate alkyl-silicas with alkyl chain lengths of 18, 8 and 1 respectively. The Supelcosil LC-18DB column has been described (24) as a state-of-the-art column for the analysis of peptides, proteins, and amino acids, employing a special silica deactivated for basic compounds. The silica is processed by new derivatization techniques to yield 5 micron spherical particles described as high carbon loaded with "capped" residual silanol groups. The Radial-Pak columns represent a new technology in

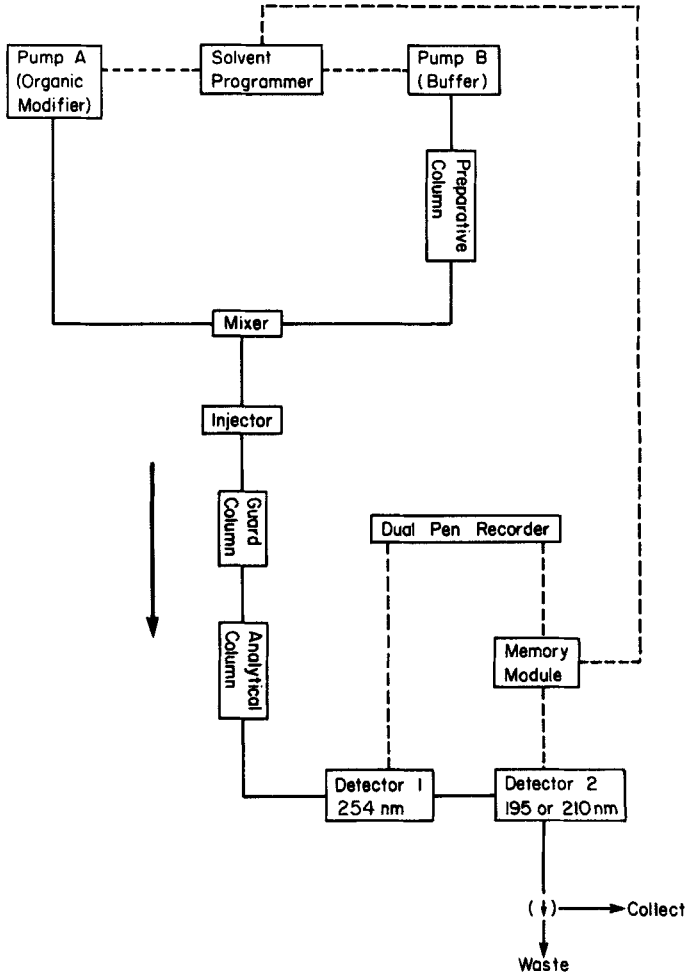

Figure 1. Schematic diagram of hplc system. Dashed lines are electrical connections.

that C_{18} microparticulate alkyl-silica is packed in a polyethylene cartridge and subjected to radial compression inside a compression chamber. The uniform pressure is said by the manufacturer to eliminate voids and channels normally present in a bed of packing material, resulting in a homogeneous chromatographic column.

TABLE 2
Buffer Systems and Hplc Conditions Used for Analysis 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)
0.01M KH_2PO_4	Acetonitrile	254, 195	90⟶ 40% B/1.0 h/ 1.1 ml/min (curve 5)
0.1% H_3PO_4	0.1% H_3PO_4 in Acetonitrile	254, 195	90⟶ 40% B/1.0 h/ 1.0 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)

The use of Radial-Pak columns for the separation of peptide and protein mixtures has recently been described by Hancock et al. (25, 26).

The four invertebrate peptide hormones were separated by almost all combinations of buffer systems and columns used in the study. Retention times are shown in Table 3. As can be seen both LAKH and CECH were almost always found to elute with 1-2 min of each other at approximately 50 mins. By contrast, retention times for proctolin and MCEN were found to vary greatly depending upon the particular system. The chromatographic behavior of these invertebrate peptides can be explained by examination of their amino acid sequences. The CECH and LAKH which have similar sequences, both contain only neutral amino acids and in addition both contain NH_2-terminal pyroglutamic acids and COOH-terminal amides. These structural features would be expected to render them relatively insensitive to the acidic and ion-pairing buffers used in this study. On the other hand proctolin and MCEN contain basic amino acids (Arg) which would be expected to render them very sensitive to acidic and ion-pairing buffers as observed.

Of all the U.V. transparent buffers tested, the TEAP buffer was clearly superior to the KH_2PO_4 and H_3PO_4 buffers on all columns,

TABLE 3
Retention Times of Four Invertebrate Neuropeptides on Various RP-Hplc Columns with Four Buffer Systems

Peptide[a]	Retention Time (min)			
	TEAP[b]	KH_2PO_4	H_3PO_4	TFA
Supelcosil LC-18DB Column[c]				
Proctolin	16.6	20.9	27.9	32.5
MCEN	31.4	43.7[d]	49.0[e]	44.4
LAKH	50.3	50.4	51.3	51.7
CECH	50.8	51.2	51.8	52.3
Zorbax C-8 Column				
Proctolin	10.1	26.6	37.1	36.2
MCEN	27.7	51.4[e]	52.3[e]	52.2
LAKH	48.3	49.4	50.0	50.9
CECH	49.9	51.0	51.7	52.9
Zorbax TMS Column				
Proctolin	f	8.9	10.2	25.3
MCEN	6.5	25.2[d]	27.0	41.9
LAKH	44.1	38.7[g]	39.4[g]	50.8
CECH	44.7	38.7	39.4	51.9
Radial-Pak C_{18}-5 mm ID Column				
Proctolin	10.9	34.9	h	49.1[d]
MCEN	27.5	h	h	h
LAKH	46.8	47.3	51.2	47.4
CECH	47.5	48.2	51.9	48.3
Radial-Pak C_{18}-8 mm ID Column				
Proctolin	12.1	35.9	h	54.4[d]
MCEN	29.3	h	h	h
LAKH	50.5	50.4	54.4	53.6
CECH	51.1	51.2	55.0	54.9

a 250 ng/25 µl .1% TFA of each peptide was injected
b Buffers and hplc conditions are described in Table 2
c Columns are described in Table 1
d Broad
e Very broad
f Eluted with solvent front
g LAKH and CECH coeluted
h Peak not observed

especially with regard to the MCEN peak which was very broad and could not be seen at all on some columns with phosphate buffers. With TEAP peaks were sharpest on the stainless steel columns (Figure 2) and varied in retention time with the chain length of the alkyl silica (Table 3). On the Zorbax TMS column proctolin eluted with the solvent front. Both the Zorbax C-8 and Supelcosil LC-18DB column gave excellent results with almost identical sensitivity. Although resolution of CECH and LAKH was better on the Zorbax C-8 column, the Supelcosil LC-18DB with TEAP buffer demonstrated excellent sensitivity and resolution of small and intermediate peptides as well as larger peptides, such as bovine, ovine, and porcine insulin (Figure 3), which might be attributed to the use of base-deactivated silica in this column. By comparison, the insulin peaks on the Zorbax C-8 column were broader and poorly resolved with TEAP. Detection limits for the invertebrate peptides are shown in Table 4 for defined conditions; as little as 2 ng of CECH could be detected.

As can be seen in Figure 4, good separations were also obtained with TEAP on the Radial-Pak columns, but the peaks were less sharp than for the stainless steel columns. As expected, the larger ID 8 mm column gave longer retention times, and the 5 mm column gave slightly better resolution. The stainless steel columns employed in this study contain packing materials that have undergone a secondary capping reaction to reduce the concentration of free silanol groups. By contrast, Hancock and Sparrow (26) have reported that the packing material in the Radial-Pak columns has a low C_{18}-loading (5% W/W) combined with the absence of secondary capping, giving rise to a mixed-mode separation mechanism of adsorption and reverse-phase because of the presence of significant concentrations of free silanol groups.

The poorer peak shapes seen by us with the Radial-Pak columns could be due to the presence of these "uncapped" silanol groups. The retention data for the basic arginine-containing peptides,

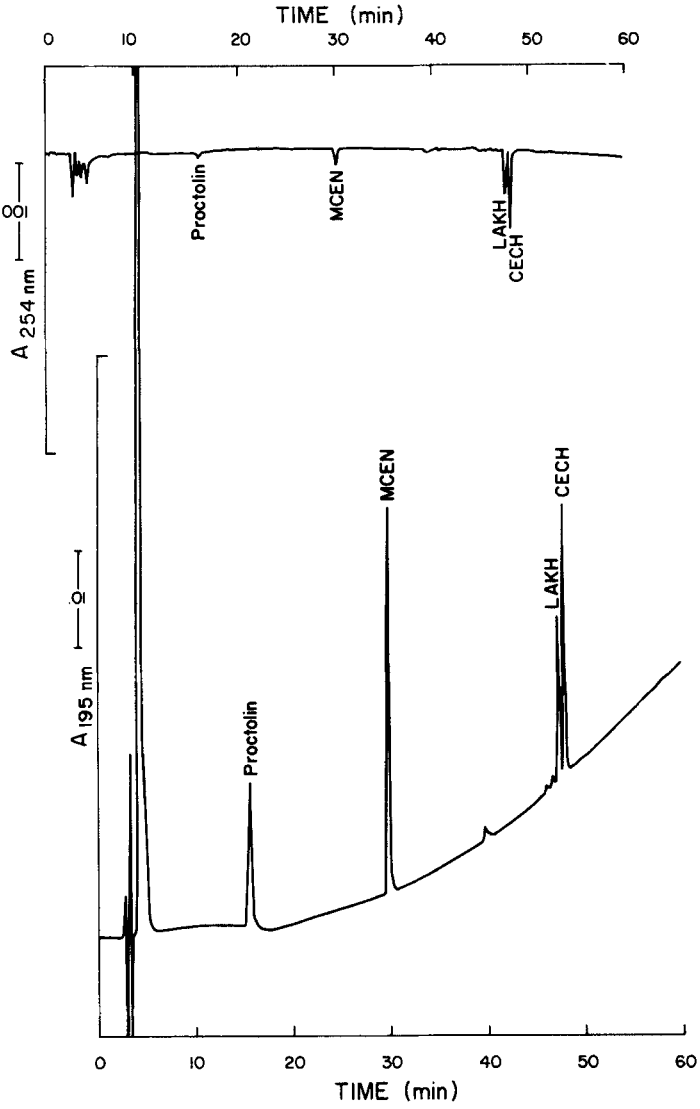

Figure 2. Analysis of four invertebrate neuropeptides with the TEAP buffer system on three stainless steel columns, Supelcosil LC-18DB (top trace), Zorbax C-8 (middle trace), and Zorbax TMS (bottom trace). Columns, buffer system and hplc conditions are fully described in Tables 1 and 2.

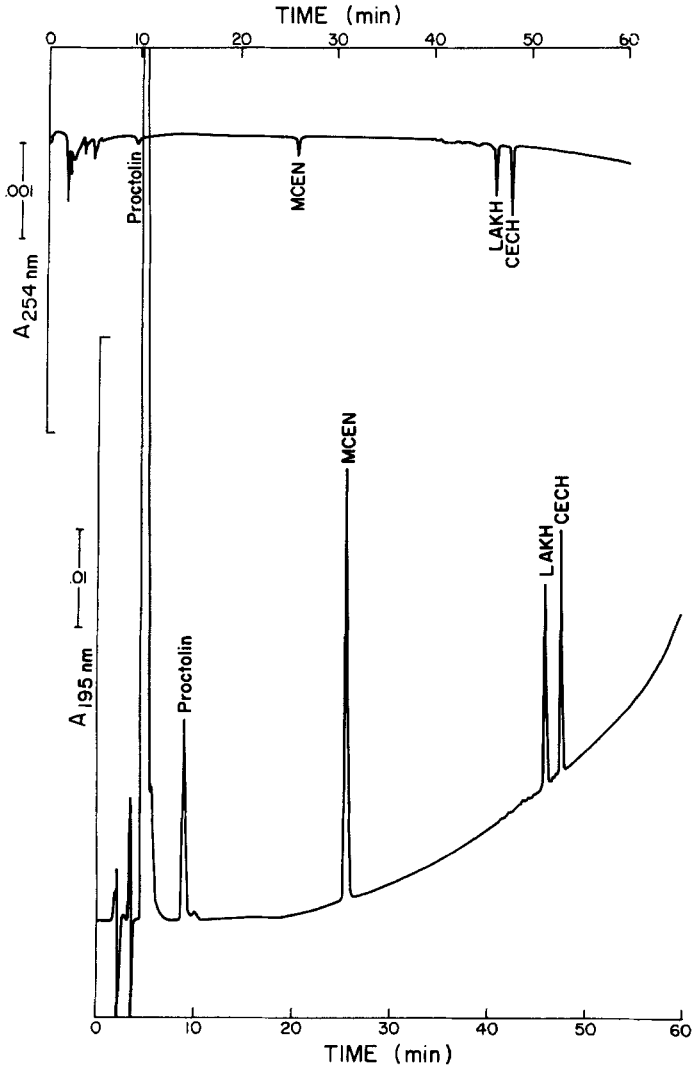

Figure 2B (middle trace)

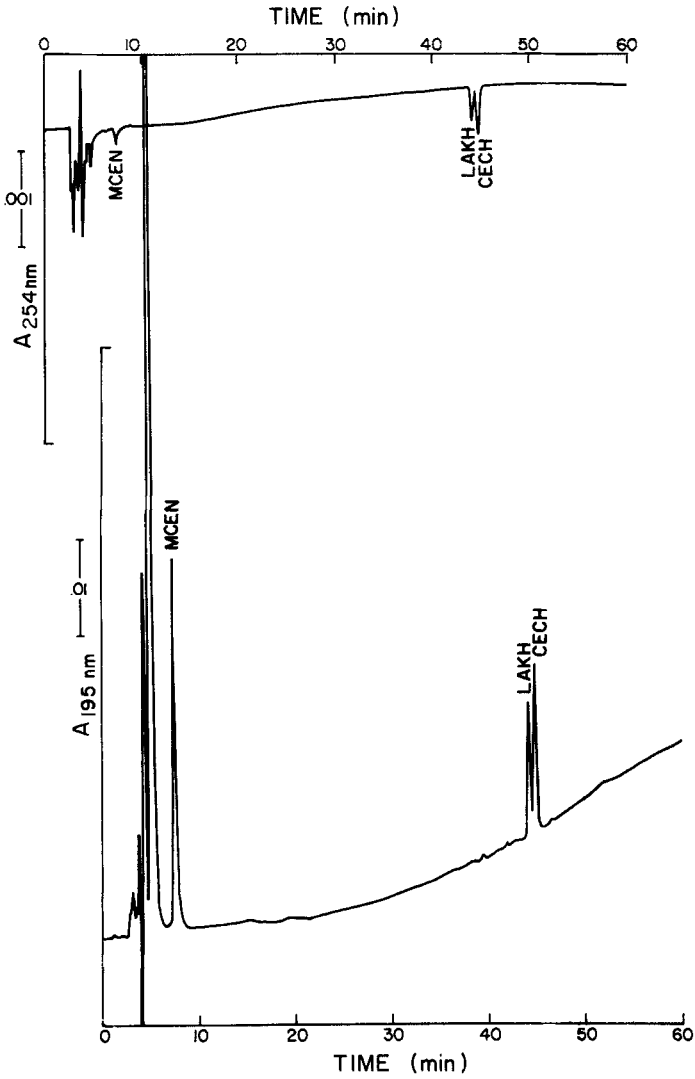

Figure 2C (bottom trace)

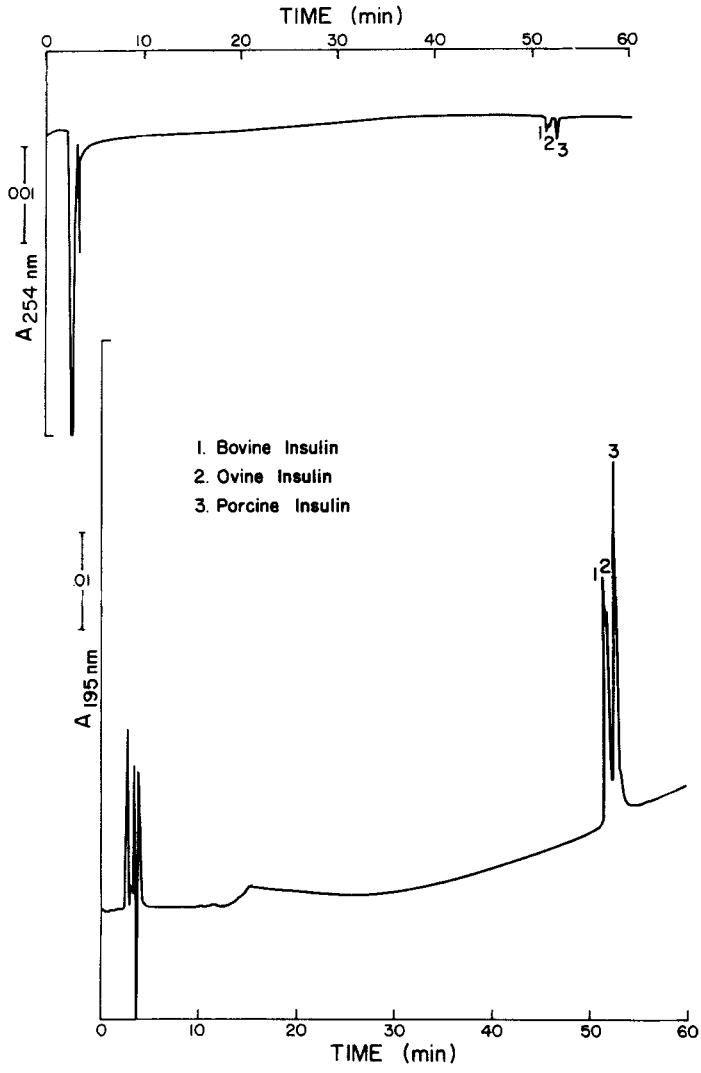

Figure 3. Analysis of bovine, ovine, and porcine insulin with the TEAP buffer system on Supelcosil LC-18DB column. Columns, buffer system and hplc conditions are fully described in Tables 1 and 2.

TABLE 4
Limits for the Detection of Invertebrate Neuropeptides on Supelcosil LC-18DB with TEAP Buffer[a]

Peptide	Injection Size (µl)	Detection Limit (ng)[b]
Proctolin	0.5	5
MCEN	0.4	4
LAKH	0.4	4
CECH	0.2	2

a Buffer and hplc conditions are shown in Table 2
b For 3:1 signal-to-noise ratio at 195 nm

proctolin and MCEN support this hypothesis. The peptide MCEN was not eluted from the Radial-Pak columns during the course of the gradient with any of the non-amine-containing buffers and proctolin exhibited significantly longer retention times with KH_2PO_4 and TFA and was not eluted at all with H_3PO_4 on the Radial-Pak columns (Table 3). Amine-containing buffers such as TEAP are known to moderate the effects of free silanol groups (27).

Because of its greater U.V. absorption, the TFA buffer required operation at 210 nm resulting in about a 50% loss of sensitivity on the Supelcosil LC-18DB column compared to TEAP as evidenced by the load-response curves for CECH (Figure 5). Nevertheless the volatility of the TFA allows its removal from collected fractions prior to performance of a bioassay. As seen in Table 3, trends observed for other buffers were observed for TFA. The stainless steel columns outperformed the Radial-Pak columns. Excellent results were obtained with both the Supelcosil LC-18DB and the Zorbax TMS columns in that all peaks were sharp and well separated. The Zorbax TMS column did demonstrate slightly better resolution of CECH and LAKH.

Retention times of the invertebrate peptides were reproducible within 1 min during the course of the several months of this study. However, as expected, there was some deterioration of the columns with use as evidenced by peak broadening, particularly in the case of the Zorbax TMS column which contains packing material whose

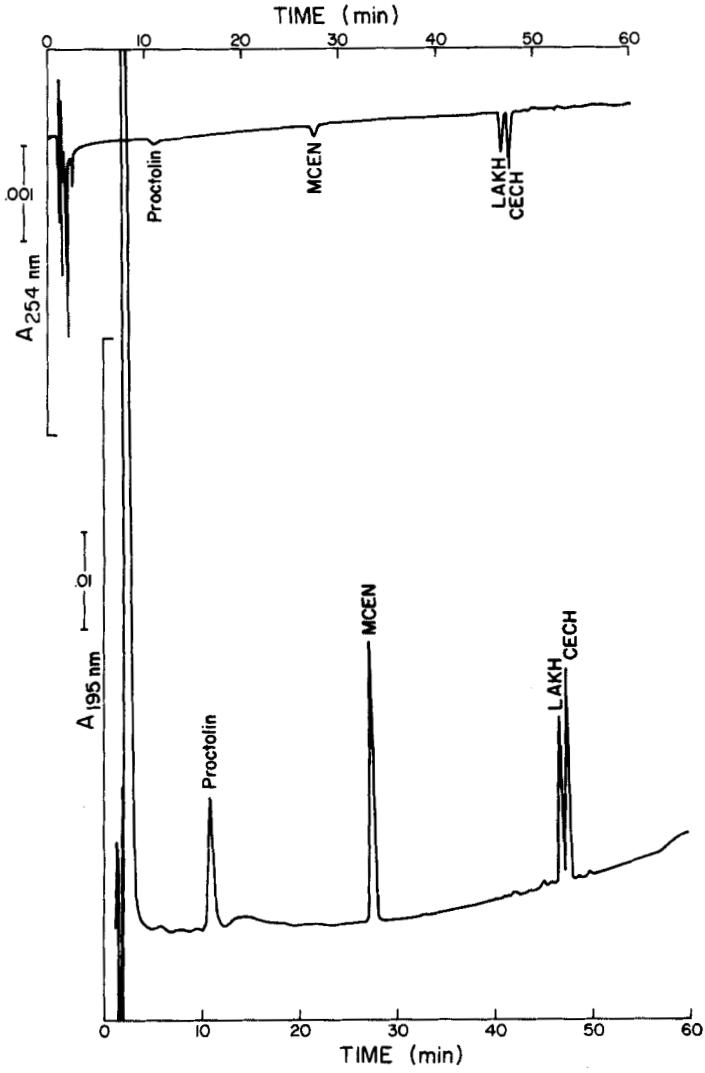

Figure 4. Analysis of four invertebrate neuropeptides with the TEAP buffer system on Radial-Pak columns, 5 mm i.d. (top trace) and 8 mm i.d. (bottom trace). Columns, buffer system and hplc conditions are fully described in Tables 1 and 2.

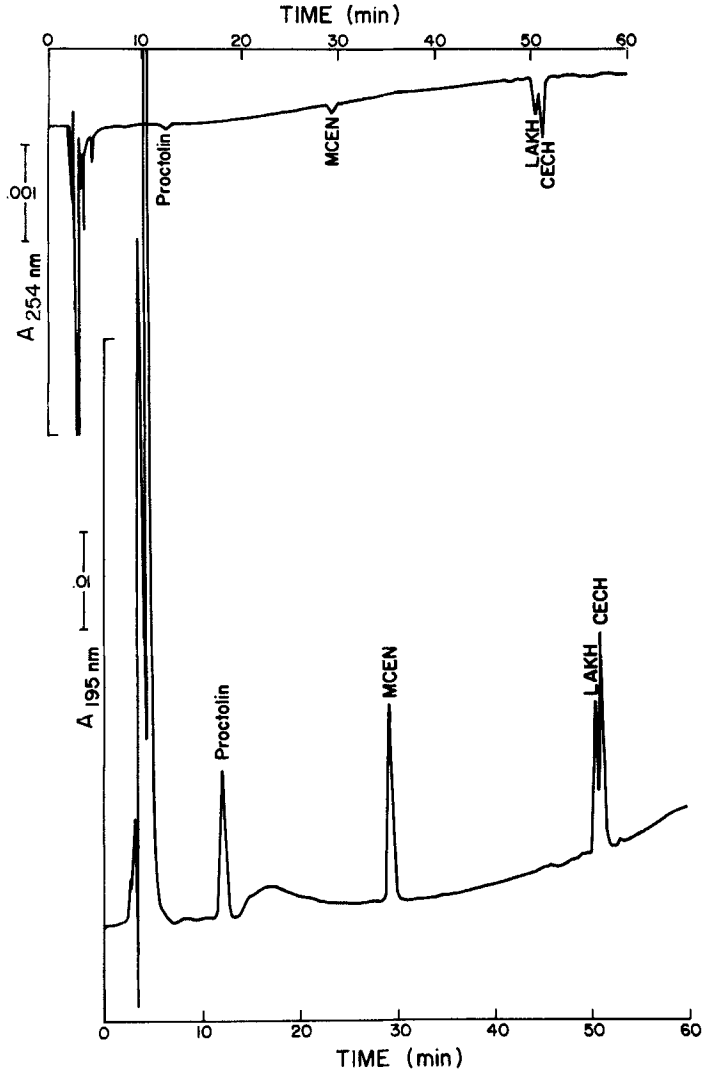

Figure 4B (bottom trace)

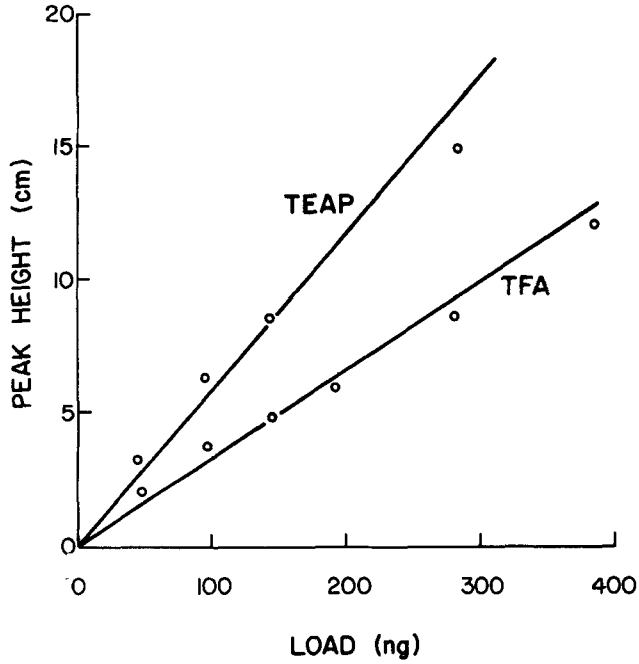

Figure 5. Load response curve for CECH on the Supelcosil LC-18DB column with the TEAP and TFA buffer systems. Columns, buffer systems and hplc conditions are fully described in Table 1 and 2.

short alkyl chains (C-1) would offer little steric hindrance to cleavage and formations of free silanol groups.

Our hplc system incorporates the Schoeffel Model MM 700 memory module (Figure 1), which can provide for baseline correction during the course of a gradient run where increasing concentration of buffer results in an increase in absorbance, particularly at low wavelengths. Although baseline shifts were not considered excessive in this study, an example of baseline correction is shown in Figure 6 for the analysis of the four invertebrate peptides with the TEAP buffer on the Supelcosil LC-18DB column.

TABLE 5

Absorbance Ratio for Invertebrate Neuropeptides on Zorbax C-8 and Supelcosil LC-18DB Columns with TEAP Buffer[a]

Peptide	Column	Absorbance Ratio[b]
Proctolin	Supelcosil LC-18DB	21.06
MCEN	Supelcosil LC-18DB	24.05
LAKH	Supelcosil LC-18DB	3.68
CECH	Supelcosil LC-18DB	3.44
Proctolin	Zorbax C-8	25.14
MCEN	Zorbax C-8	21.90
LAKH	Zorbax C-8	3.79
CECH	Zorbax C-8	3.37

a Buffer and hplc conditions are described in Table 2
b $A_{195\ nm}/A_{254\ nm}$

By simultaneous monitoring of two wavelengths, 254 and 195 (for TEAP, KH_2PO_4 and H_3PO_4) or 210 (for TFA), we were able to measure absorbance ratios for the invertebrate peptides. Absorbance ratios can be used to provide positive confirmation of hplc peaks (28). Absorbance ratios for the four invertebrate peptides on the Zorbax C-8 and Supelcosil LC-18DB columns with TEAP buffer are shown in Table 5.

In summary, in the analysis of four invertebrate neuropeptides by hplc excellent sensitivity and resolution were observed with the TEAP buffer on both the Zorbax C-8 and Supelcosil LC-18DB columns where the detection of as little as 2 ng of peptide was possible.

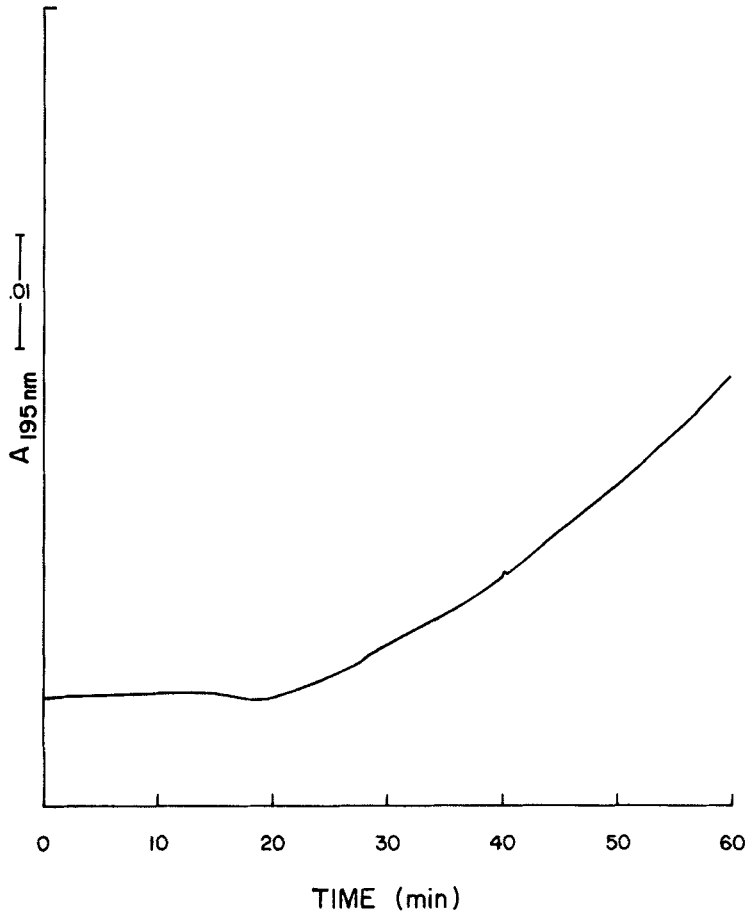

Figure 6. Baseline corrections at 195 nm during the analysis of four invertebrate neuropeptides with the TEAP buffer on the Supelcosil LC-18DB column, uncorrected baseline (top trace) and corrected chromatograph (bottom trace). Column, buffer system and hplc conditions are fully described in Tables 1 and 2.

Of the systems examined, the Supelcosil LC-18DB column with TEAP buffer gave better resolution for the insulins. The volatile TFA buffer demonstrated lower sensitivity with excellent results observed on both the Supelcosil LC-18DB and Zorbax TMS columns.

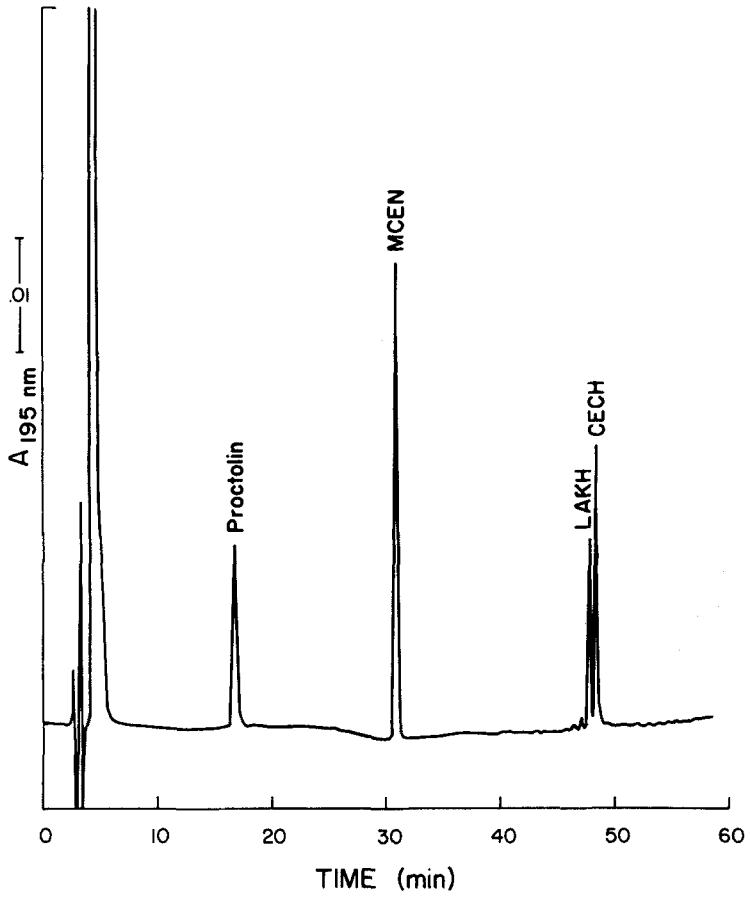

Figure 6B (bottom trace)

We feel that this study clearly demonstrates the value of reverse phase hplc for the analysis of nanogram amounts of invertebrate neuropeptides. The application of these results in the analysis and isolation of invertebrate neuropeptides from small tissue samples is currently under investigation.

ACKNOWLEDGMENT

We would like to thank Kenneth W. Young for his efforts in operating the hplc equipment.

FOOTNOTES

1. Mention of a commercial product in this paper does not constitute an endorsement of this product by the USDA.

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