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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: PREPARATIVE PURIFICATION OF SYNTHETIC PEPTIDES*

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SUMMARY

Biologically active peptides synthesized by the solid phase methodology of Merrifield were purified by reversed-phase high-performance liquid chromatography using newly developed preparative radially compressed cartridges fitting Waters Assoc. Prep LC 500 liquid chromatograph. Cartridges were handpacked with Vydac C_{18}, C_4 or diphenyl derivatized silicas (pore size 300 Å) of different particle sizes (10–20 μm). Large scale purification of gram amounts of gonadotropin releasing hormone analogs (agonist and antagonist) as well as amidated human pancreatic tumor growth hormone releasing factor (a 40-peptide) illustrate the resolutive power of this technique applied to the isolation of more than 300 synthetic peptides in our laboratory over the last two years. Difficult separations were achieved by changing supports (C_{18}, C_4, diphenyl) as well as mobile phase composition: (triethylammonium phosphate pH 2.25 or 6.5, 0.1% trifluoroacetic acid, ammonium acetate pH 6.5 and acetonitrile). Protected amino acids and peptides amenable to normal-phase chromatography on Vydac spherical underivatized silica were purified economically by the reversed-phase mode. It is understood that this general, convenient and versatile strategy may be applicable to the preparative scale isolation of any other class of compounds usually separated on reversed-phase high-performance liquid chromatography.

INTRODUCTION

Extrapolation from analytical to semipreparative and, finally, to preparative (gram amount) separations of small biologically active peptides from nine to fourteen amino acids (gonadotropin releasing hormone and analogues, and somatostatin) has been shown possible and has been performed routinely in our laboratory for several years using stainless-steel 30 × 1 cm I.D. C_{18} columns from Waters Assoc.[1,2] or

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radially compressed Prep Pak C_{18} cartridges from Waters Assoc. with the Prep LC-500 apparatus[3]. Similar results dealing with small protected or unprotected peptides have also been reported by others[4–8], but the limits of the methodology were already apparent: (a) lack of flexibility due to the unavailability of gradient capabilities of the instrumentation; (b) poor mechanical as well as inadequate chromatographic characteristics for peptides of the reversed-phase C_{18} silica in the commercially available cartridges. This is without mentioning that large quantities (gram amounts) of small peptides are costly, thus limiting the number of trial runs which could be performed. The first convincing evidence, however, that preparative high-performance liquid chromatography (HPLC) had great potential was that even with these drawbacks, it performed better on somatostatin than the then only available preparative technique (*i.e.*, counter current distribution) that could theoretically be scaled up[3].

Concomitantly we analysed solid phase-generated crude synthetic preparations of peptides 15–40 amino acids long which were considerably more complex than those of shorter ones; furthermore recovery of the 15- to 40-peptides from traditional reversed-phase packings was often poor. In search for better chromatographic supports we could demonstrate the positive effects of further developments in analytical column technology as illustrated in the isolation of naturally occurring polypeptides such as ovine CRF[9]. Similarly we reported on the importance of the support in the separation of 10- to 51-peptides[10] and concluded that pore size (300 Å *versus* 80–120 Å) played an important, if not major, role in the chromatographic process (recovery and peak symmetry). Later we examined the effect of different Vydac silicas (300 Å, 5-μm particle size) and solvents on the analytical separation of insulins[11]. Here we report on a preparative approach (gram amount) to the purification of synthetic peptides (10–44 residues) generated by the Merrifield solid phase technique[12].

MATERIALS AND METHODS

Apparatus

The analytical chromatographic systems consisted of:

(1) Waters Assoc.: two 6000A pumps; Data Module recorder and integrator; Wisp 710B, automated sample injector; System Controller 720 programmer and Kratos Spectroflow 773 variable-wavelength UV detector.

(2) Waters Assoc.: two M-45 pumps and an Automated Gradient Controller; Houston Instruments Omniscribe strip chart recorder; Shimadzu Chromatopak-EIA integrator; Rheodyne 7125 injector and a Kratos SF 769Z variable-wavelength UV detector.

(3) Altex: two Model 100A pumps; 420 System Controller; 400 mixing chamber; Houston Instruments Omniscribe strip chart recorder; Shimadzu Chromatopak-EIA integrator; Rheodyne 7125 injector and a Waters Assoc. 450 variable-wavelength UV detector.

The preparative chromatographic systems consisted of:

(1) Waters Assoc.: Prep LC/System 500 which was altered to allow flow-rate increments of 25 ml/min; 450 variable-wavelength UV dectector; Linear instruments Model 455 strip chart recorder and an Eldex Laboratories Chromat-A-Trol Model II gradient maker.

(2) Waters Assoc.: Prep LC/System 500A which was altered to allow flow-rate increments of 25 ml/min; Perkin-Elmer LC-75 UV variable-wavelength detector; Eldex Laboratories Chromat-A-Trol Model II gradient maker and a Curken Model 250-2A strip chart recorder.

Analytical columns

The analytical columns were the Vydac 5-μm, 300-Å columns reported earlier[11].

Preparative cartridges

Empty polyethylene cartridges and frits (part numbers 50411 and 50421) obtained from Waters Assoc. were dry-packed in our laboratory with Vydac C_{18}, C_4 and diphenyl derivatized silicas (The Separations Group, Hesperia, CA, U.S.A.) having varying particles sizes; 10 μm, 15 μm, 17 μm, 20 μm and 15–20 μm. A well packed cartridge is one that would be only barely deformed after radial compression; it will contain 460 ± 3 g of C_{18} 15–20-μm silica (218TPB1520) (measured on two cartridges filled with the same batch of silica). Solvent content or density of the silicas may however vary from batch to batch and result in variable weights of the final cartridges. Whereas 10-μm particles would not allow for flow-rates greater than 50 ml/min (with a limiting back pressure of 35 atm), cartridges packed with 15-μm, 17-μm and 20-μm particles would allow for flow-rates of 75–125 ml/min which were found adequate since it corresponded to *ca.* half the linear flow used routinely on analytical columns. Rather than using 15-, 17- or 20-μm particles which are expensive because of the rigorous sizing process, we found later that the 15–20-μm materials, while relatively inexpensive, were ideally suited as packing materials for cartridges subjected to radial compressions. Cartridges packed with Vydac C_{18} silica: 30 μm (packed by Waters Assoc.) and 40–90-μm particle sizes were also tested and found to be significantly less resolutive than cartridges similarly packed with 15–20-μm Vydac C_{18}. The outstanding characteristics of the Vydac material were that the particles are spherical, the pore size is 300 Å and high carbon loading is followed by thorough end-capping, rendering this product particularly durable. Indeed more than 500 l of such solvents as triethylammonium phosphate (TEAP)[15] or 0.1% trifluoro acetic acid (TFA)–acetonitrile[16] have been run through some cartridges over the last two years with limited deterioration as tested by the slow appearance of free silanol groups. Well packed cartridges were particularly resistant to compression because of the spherical nature of the particles; as a result the plastic sleeves were much less stressed and withstood more than 100 compressions. In order to obtain flow-rates in the range of 100–125 ml/min, it was found that when using the 15–20-μm silicas, optimal radial compression was between 37 and 40 atm. Higher compression than 40 atm often resulted in increased back pressures which limited flow-rates to less than 75 ml/min. Back pressure was also caused by the slow clogging of both the frit and top of cartridge by solid (colloidal) matter or other non-eluable materials generally introduced with the crude peptide sample rather than with the eluting buffers. When that occurred, the frit was cut out, the colored top 2–4 cm of the cartridge replaced by new silica thus generating a new cartridge. An even more drastic step consisted of emptying the cartridge and extensively washing the silica with methanol on a Buchner funnel (Whatman 1 qualitative paper). The silica was then dried at 50°C in

a vacuum oven and repacked in a new sleeve. These repacked cartridges however generally did not perform as well as the original ones. This may have been due to the appearance of silanols and fines. A cartridge was also packed with Vydac spherical underivatized silica (15–20 μm) using the same technique in order to test its usefulness for protected amino acids and peptides.

Solvent systems

The TEAP 2.25 and the 0.1% TFA buffer systems have been described earlier[15,16]. The TEAP 6.5 buffer was made from 0.1% phosphoric acid (v/v), 0.28% (v/v) triethylamine. The 6.5 ammonium acetate buffer[1] was 0.1 *N* ammonium acetate adjusted to pH 6.5 with ammonium hydroxide or acetic acid. Columns or cartridges, solvents, flow-rates and loads are described in the figure legends illustrating each of the different chromatographic separations. Solvent A was always the aqueous buffer and solvent B was either 60% acetonitrile, 40% A in the case of the TEAP 2.25 buffer and ammonium acetate 6.5 buffer or in the case of 0.1% TFA and TEAP 6.5 buffers: 0.1% TFA in acetonitrite–water (60:40) and 0.1% phosphoric acid, 0.28% triethylamine in acetonitrile–water (60:40). The wavelength at which the eluent was monitored is shown on the ordinate, gradient shape is shown by a dotted line.

Peptides

Peptides with the exception of insulin (a generous gift from Eli Lilly) were synthesized by the solid phase approach on a Beckman 990B synthesizer when amounts smaller than 1 g of purified peptide were desired and manually for amounts up to 25 g of final product. The general approach was that described by Märki *et al.*[17]. In short, peptides up to 44 residues were assembled on either chloromethylated or methyl benzhydrylamine resins depending on whether the desired final product had a C-terminus carboxylic acid or carboxamide. Hydrogen fluoride cleavage and deprotection in the presence of scavengers yielded, after lyophilization, the crude peptidic preparations which were submitted to preparative chromatography. Biologically active peptides that were purified in that manner included: GnRH and analogues, vasopressin and analogues (antagonists), neurotensin, bombesin and sub-

TABLE I

PRIMARY STRUCTURES OF PEPTIDES

	Peptide	*Column, buffers, gradient**
CCK-8:	Cholecystokinin octapeptide: [Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2] MW 1143	A, D, 18%–60′–38%B
GnRH:	Gonadotropin releasing hormone: (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) MW 1182	A, C, 19%(2′)–60′–35%B
	[DTrp^6,Pro^9-NEt]-GnRH	A, C. 30%(2′)–40′–50%B
	[N^{im}BzlDHis^6,Pro^9-NEt]-GnRH	A, C, 16%(2′)–60′–32%B
	[Ac-ΔPro^1,4FDPhe^2,DTrp^{3,6}]-GnRH	A, C, 50%(2′)–45′–75%B
	[Ac-DNal(2)^1,4FDPhe^2,DTrp^3,DArg^6]-GnRH	A, C, 40%–60′–70%B
Caerulin:	(pGlu-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2) MW 1353	A, D, 5%–60′–25%B
SP:	Substance P: (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) MW 1348	A, C, E
BN:	Bombesin: (pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-	A, C, 23%–60′–43%B

TABLE I (*continued*)

	Peptide	*Column, buffers, gradient**
	Met-NH_2) MW 1620	
NT:	Neurotensin: (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) MW 1673	A, C. E
SS-14:	Somatostatin: (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH) MW 1638	A, C, E
GRP:	Gastrin releasing peptide: (Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) MW 2785	A, C, 27%–60′–46%B
PHI:	Peptide [His^1, Ile^{27}-NH_2]: (His-Ala-Asp-Gly-Val-Phe-Thr-Ser-Asp-Phe-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys-Lys-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2) MW 2991	A, C, 42%–80′–62%B
SS-28:	Somatostatin-28: (Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH) MW 3137	A, C, 24%(4′)–50′–40%B
VIP:	Vasoactive intestinal peptide: (His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2) MW 3326	A, C, 35%–60′–60%B
βh-End:	β-Human endorphin: (Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH) MW 3465	A, C, 19%(2′)–60′–38%B
CGRP:	Calcitonin gene related peptide: (Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-Phe-NH_2) MW 3801	B, C, 5%(5′)–60′–40%B
hpGRF:	Human pancreatic (tumor) growth hormone releasing factor: (Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-OH) MW 4538	A, C, 35%–60′–55%B
	[NLe^{27}]hpGRF$^{1-29}$-NH_2	A, C, 40%–60′-65%B
oCRF:	Ovine corticotropin releasing factor: (Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2) MW 4670	A, C, 43%–60′–63%B
Sauvagine:	(pGlu-Gly-Pro-Pro-Ile-Ser-Ile-Asp-Leu-Ser-Leu-Glu-Leu-Leu-Arg-Lys-Met-Ile-Glu-Ile-Glu-Lys-Gln-Glu-Lys-Glu-Lys-Gln-Gln-Ala-Ala-Asn-Asn-Arg-Leu-Leu-Leu-Asp-Thr-Ile-NH_2) MW 3780	A, C, 43%–60′–62%B
Carp urotensin:	(Asn-Asp-Asp-Pro-Pro-Ile-Ser-Ile-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Asn-Met-Ile-Glu-Met-Ala-Arg-Asn-Glu-Asn-Gln-Arg-Glu-Gln-Ala-Gly-Leu-Asn-Arg-Lys-Tyr-Leu-Asp-Glu-Val-NH_2) MW 4880	A, C, 38%–60′–58%B
Sucker urotensin:	(Asn-Asp-Asp-Pro-Pro-Ile-Ser-Ile-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Asn-Met-Ile-Glu-Met-Ala-Arg-Ile-Glu-Asn-Glu-Arg-Glu-Gln-Ala-Gly-Leu-Asn-Arg-Lys-Tyr-Leu-Asp-Glu-Val-NH_2) MW 4871	A, C, 45%–60′–65%B
rACTH:	Rat adrenocorticotropin: (Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Val-Ala-Glu-Asn-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH) MW 4761	A, C, 25%–60′–45%B
Porcine insulin:	A-Chain: (Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Gln-Asn-Tyr-Cys-Asn-OH) B-Chain: (Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-OH) MW 5722	A, C, E

* A = C_{18} 300 Å Vydac; B = C_4 300 Å Vydac; C = TEAP 2.25 = A; 60% acetonitrile–A (60:40) = B; D = 0.1 *M* ammonium acetate, pH 6.5 = A; 0.1 *M* ammonium acetate–acetonitrile (40:60) = B; E = see legend to Fig. 1.

stance P, somotastatin-14 and -28 and analogues, GRP and fragments[18], CRFs (ovine and rat), its homologues (sauvagine and urotensin I) as well as analogues[19], GRFs (human and rat) and analogues[13,20] and members of that family (VIP and PHI, unpublished results), CGRP and fragments[21], CCK-8 analogues[22] and caerulein[23], endorphins, rat ACTH (unpublished) and conotoxins[24,25]. Selected peptide sequences and chromatographic conditions are reported in Table I. More than 1500 individual preparative runs were performed over a period of two years using the technique reported here.

Finally, this technique played a crucial role in our ability to purify rat hypothalamic CRF and GRF from crude tissue extracts after they had been subjected to gel permeation[14,20].

Sample preparation and loading

The crude peptide was generally dissolved in a buffer whose strength in organic modifier was equal to or below that used for the equilibration of the cartridge prior to the run and was filtered through a 5-μm filter, ACRO™ 50A (Gelman Sciences). The solution was then loaded onto the column through the pumps and chromatographed using gradient conditions (see below).

Technical inconveniences

Two problems associated with our particular instruments are worth mentioning. (a) Because of the fact that UV monitoring derived from a flow splitter it happened that the UV detector read either ahead or behind what actually eluted off the main line used for collection: early reading from our set up is best illustrated in Figs. 6 and 7. In Fig. 6, fractions 1 and 2 were made almost evenly (see slashes on the chromatogram which indicates at which time fractions were made) and yet the majority of the peptide was in fraction 2 (see Fig. 7). (b) The preparative systems which were used had a strong tendency, despite degassing of the solvents, of generating bubbles which interfered with UV detection. This is illustrated by the peaks marked X in Fig. 13 or A.B. in Fig. 17. Flow restriction in the gradient maker is the most likely explanation for that problem, even though we may not exclude cavitation effects in the pumping system.

EXPERIMENTAL

Fig. 1 and legend illustrate an analytical separation of several biologically active peptides on a preparative cartridge using a linear gradient of increasing concentration of acetonitrile.

Purification of a GnRH antagonist [Ac-DNal(2)^1,4FDPhe^2, (for)DTrp^3, DArg^6]-GnRH

The gradient analysis of the crude GnRH antagonist (Fig. 2) was used to assess the purity of the peptide, determine the conditions for isocratic analysis (Fig. 3) and the gradient purification (Fig. 4). The isocratic analyses were fast, less than 10 min, and allowed for the expedient analysis of the collected fractions (7 to 12) from the preparative run (*i.e.* within the 60–90 min of a preparative run) thus promptly providing the information needed for the appropriate pooling of fractions of comparable

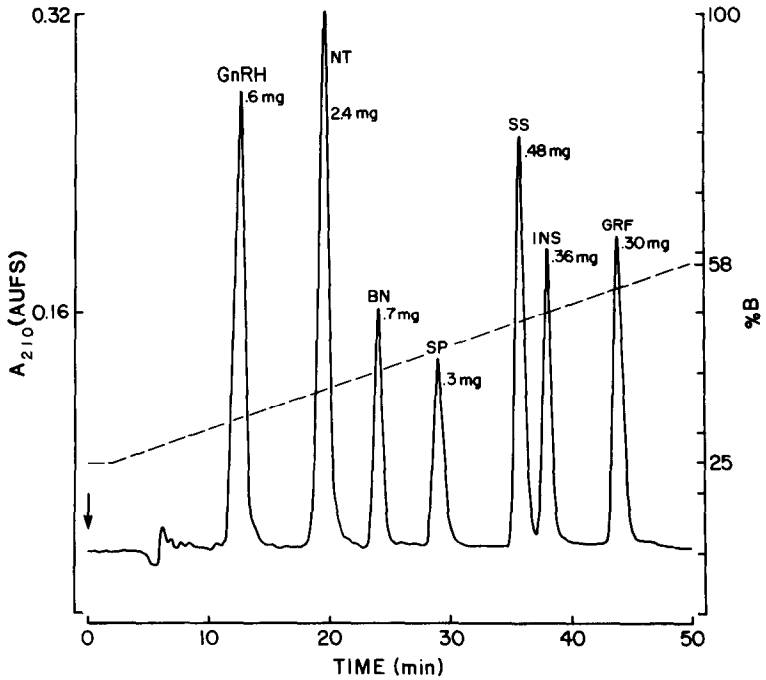


Fig. 1. Load: HPLC purified peptides as stated, structures shown in Table I. Column: PrepPak 500, 30 × 5 cm I.D., packed with Vydac 15–20 μm C_{18} material, 46 atm radial compression. Buffers: TEAP 2.25 = A; 60% acetonitrile in A = B. Gradient: 26%(2')–50'–58%B. Flow-rate: 100 ml/min, 26 atm back pressure. Detector: 0.32 (210 nm). Chart speed: 0.5 cm/min.

purity and their further processing. Both analyses were run in TEAP 2.25–acetonitrile buffer on a 5-μm C_{18} Vydac column and detected at 210 nm. The gradient preparative run of 1.2 g of this GnRH anatagonist in TEAP 2.25–acetonitrile on a 17-μm C_{18} column, detected at 285 nm is illustrated in Fig. 4. After isocratic analysis, the cuts from the main peak of the preparative run (Fig. 5) fractions 7 and 12 were pooled, diluted with an equal volume of water, and desalted in 0.1% TFA–acetonitrile on the same cartridge (Fig. 6); three fractions were collected and analysed (Fig. 7). Fractions 8–11 were pooled and desalted (Fig. 8) in 0.1% TFA–acetonitile using the same conditions as those used in desalting the pooled fractions 7 and 12, since we had achieved a good separation of the main peak from a hydrophobic impurity. The four fractions taken from this desalting step were analysed (Fig. 9). Fractions of equivalent purity (2 in Fig. 7 and 1 in Fig. 9) were pooled and lyophylized to yield 56 mg of a *ca.* 95% pure peptide (percentage purity is defined as the ratio of the integrated area of a peak over that of total integrated areas excluding loading artifact when detection is at 210 nm). Fractions 2 and 3 from the second desalting (Fig. 9) were pooled, lyophylized and analysed (Fig. 10), yielding 128 mg of a 99.7% pure peptide. Total recovery of peptide was 184 mg corresponding to a yield of 15% which compares favorably with what one had expected from the ratio of integrated area of the main peak over total area in the crude peptide, Fig. 2 (*i.e.* 22%).

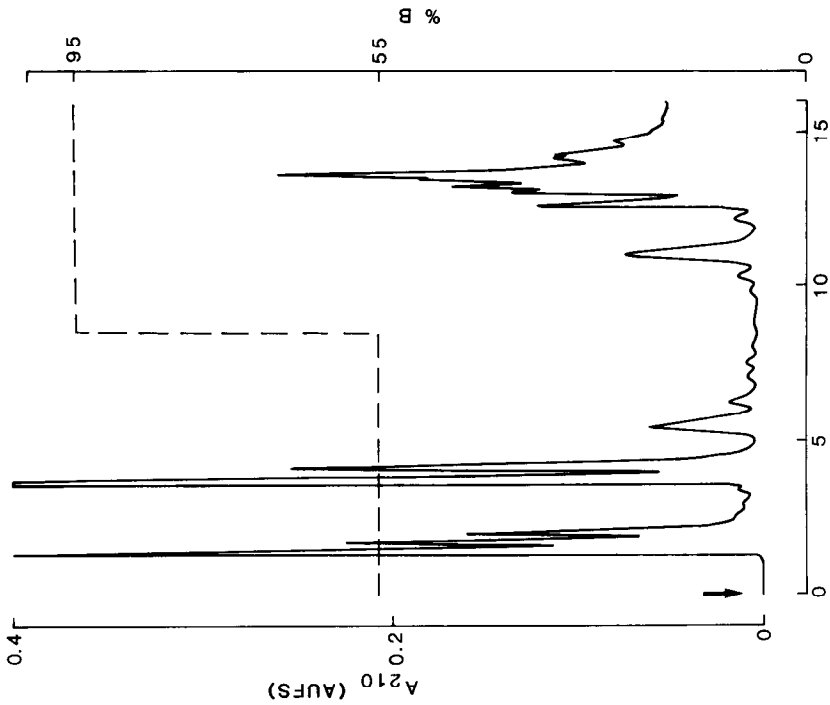


Fig. 2. Load: lyophilized [Ac-DNal(2)^1,4FDPhe^2,(for)DTrp^3,DArg^6]-GnRH (20 μl *ca.* 20 μg) from HF cleavage, *cf.* Table I for structure. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: TEAP 2.25 = A; 60% acetonitrile in A = B. Gradient: 45%–30'–95% B. Flow-rate: 2.0 ml/min, 2950 p.s.i. back pressure. Detector: 0.4 (210 nm). Chart speed: 1 cm/min.

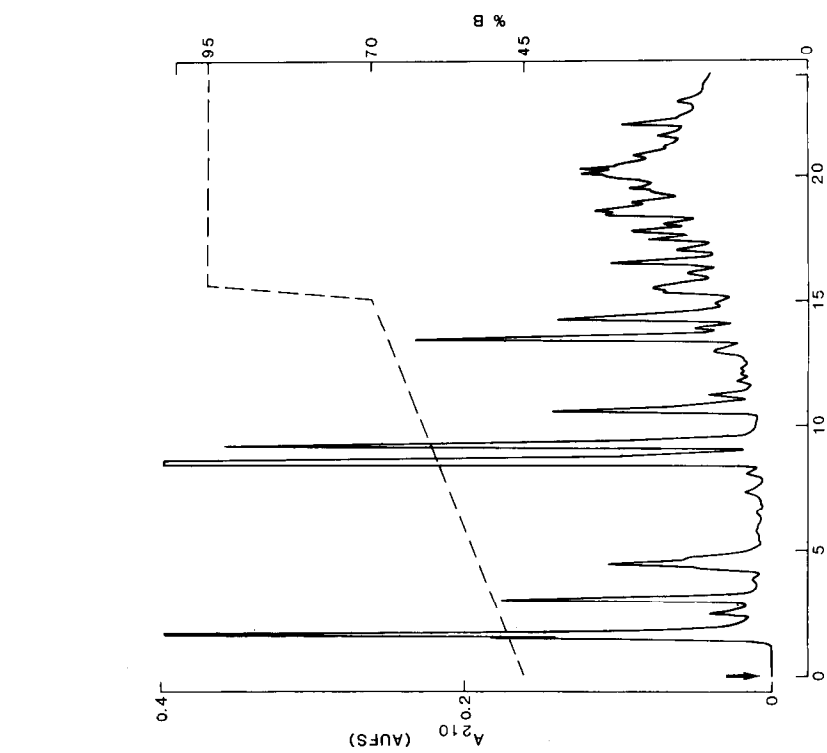


Fig. 3. Load: lyophilized [Ac-DNal(2)^1,4FDPhe^2,(for)DTrp^3,DArg^6]-GnRH (20 μl, *ca.* 20 μg) from HF cleavage. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: TEAP 2.25 = A; 60% acetonitrile in A = B. Gradient: 55%(18')–0.1'–95% B. Flow-rate: 2.0 ml/min, 2950 p.s.i. back pressure. Detector: 0.4 (210 nm). Chart speed: 1 cm/min.

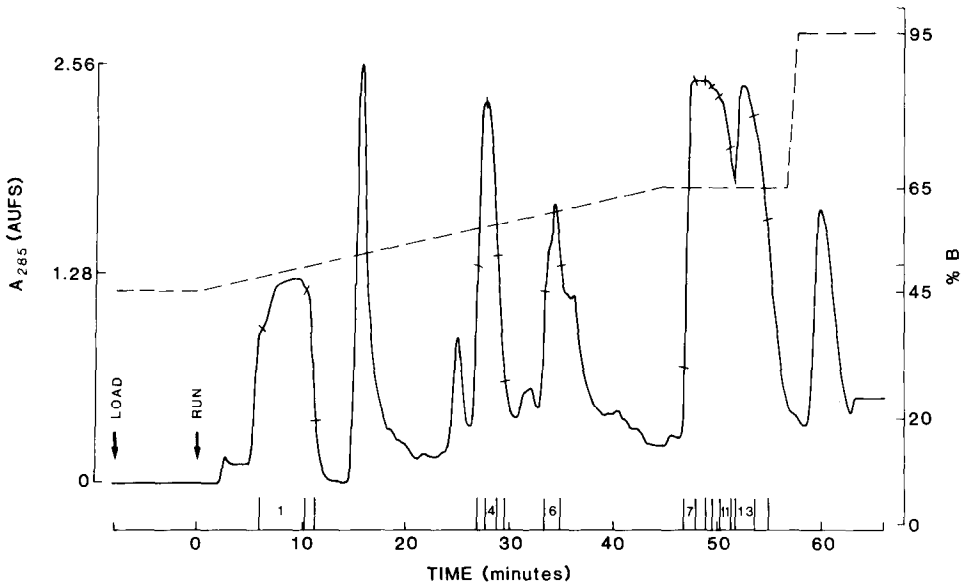


Fig. 4. Load: lyophilized [Ac-DNal(2)[1],4FDPhe[2],(for)DTrp[3],DArg[6]]-GnRH (450 ml, 1.2 g) from HF cleavage. Column: PrepPak 500, 30 × 5 cm I.D., packed with Vydac 17 μm C_{18} material, 48 atm radial compression. Buffers: TEAP 2.25 = A; 60% acetonitrile in A = B. Gradient: 45%(load)-45'-65%(12')-1'-95% B. Flow-rate: 75 ml/min, 33 atm back pressure. Detector: 2.56 (285 nm). Chart speed: 0.5 cm/min.

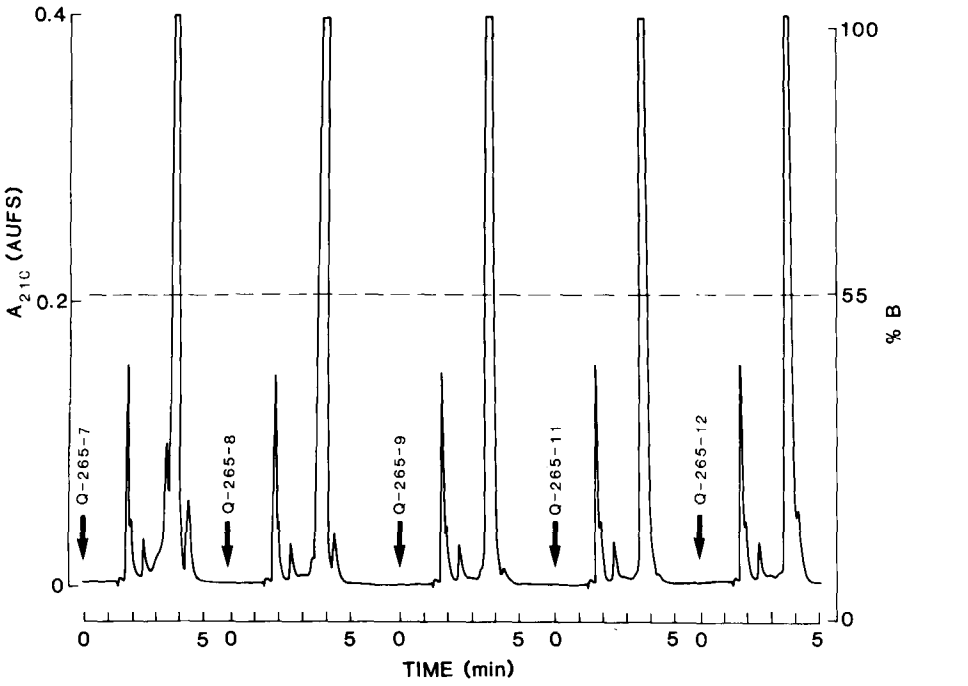
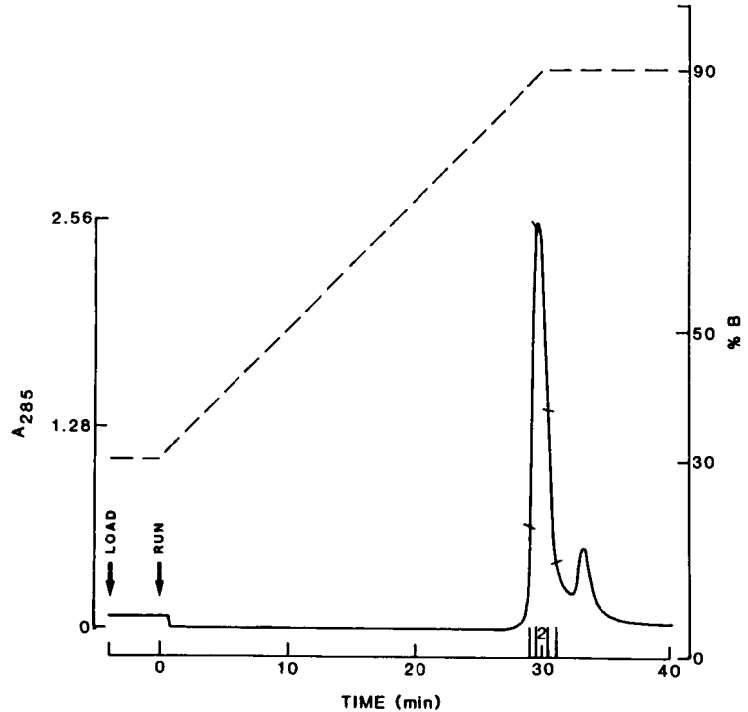


Fig. 5. Load: [Ac-DNal(2)[1],4FDPhe[2],(for)DTrp[3],DArg[6]]-GnRH fractions 7-9, 11 and 12 (20 μl, *ca.* 20 μg) from preparative purification shown in Fig. 4. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: TEAP 2.25 = A; 60% acetonitrile in A = B. Gradient: 55% B isocratic. Flow-rate: 2.0 ml/min, 2950 p.s.i. back pressure. Detector: 0.4 (210 nm). Chart speed: 1 cm/min.



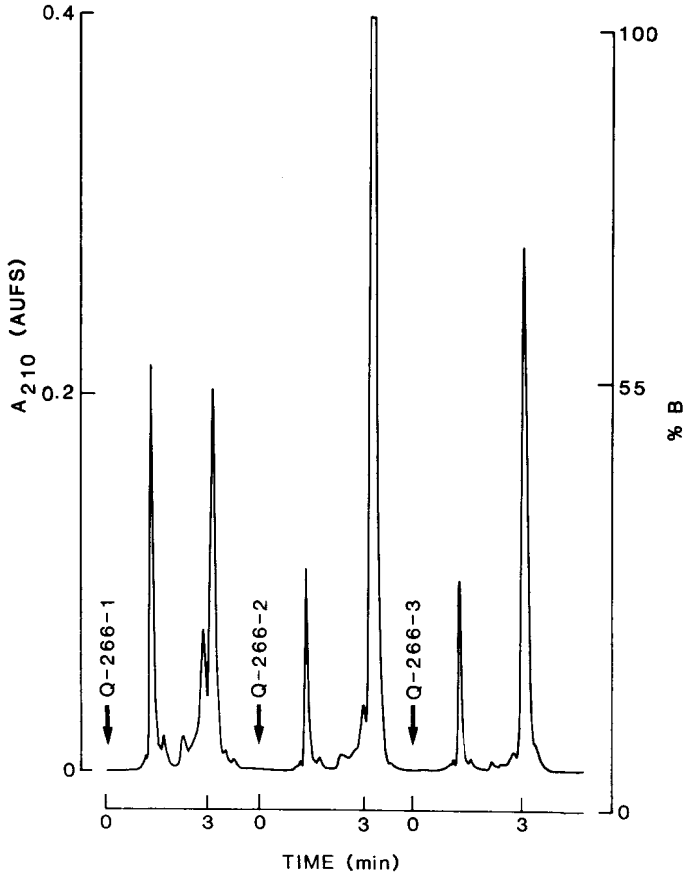


Fig. 6. Load: [Ac-DNal(2)[1],4FDPhe[2],(for)DTrp[3],DArg[6]]-GnRH (200 ml, *ca.* 0.1 g) fractions 7 and 12 from preparative purification shown in Figs. 4 and 5 for analyses. Column: PrepPak 500, 30 × 5 cm I.D., packed with 17 μm C_{18} material, 48 atm radial compression. Buffers: 0.1%TFA in water = A; 0.1%TFA in acetonitrile–water (60:40) = B. Gradient: 30%(load)–30'–90% B. Flow-rate: 75 ml/min, 33 atm back pressure. Detector: 2.56 (285 nm). Chart speed: 0.5 cm/min.

Fig. 7. Load: [Ac-DNal(2)[1],4FDPhe[2],(for)DTrp[3],DArg[6]]-GnRH fractions 1–3 (20 μl, *ca.* 5–20 μg) from preparative desalting shown in Fig. 6. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: TEAP 2.25 = A; 60% acetonitrile in A = B. Gradient: 55% B isocratic. Flow-rate: 2.0 ml/min, 2950 p.s.i. back pressure. Detector: 0.4 (210 nm). Chart speed: 1 cm/min.

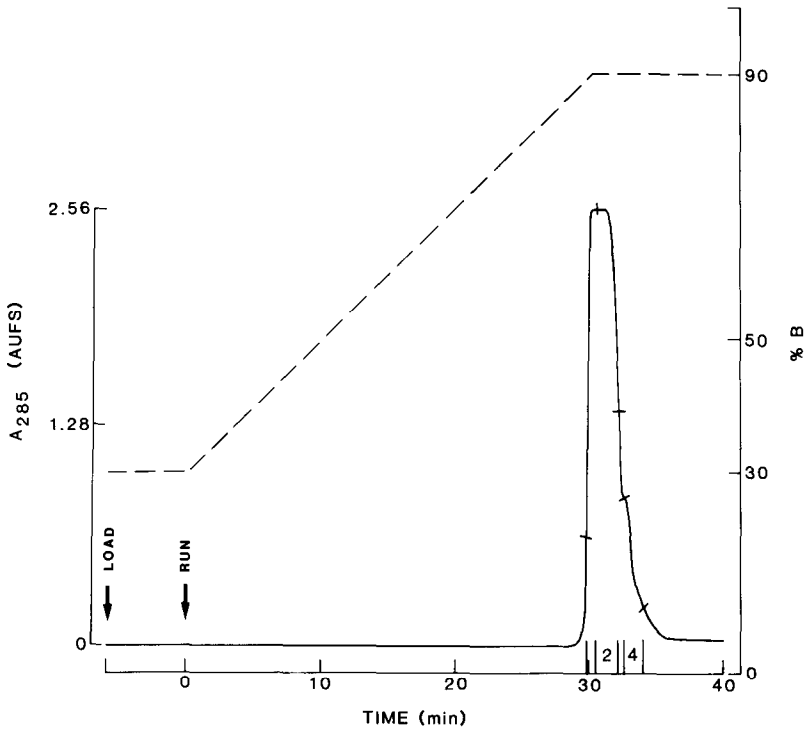


Fig. 8. Load: [Ac-DNal(2)[1],4FDPhe[2],(for)DTrp[3],DArg[6]]-GnRH, fractions 8–11 (300 ml, *ca.* 0.2 g) from preparative purification shown in Figs. 4 and 5 for analyses. Column: PrepPak 500, 30 × 5 cm I.D., packed with 17 μm C_{18} material, 48 atm radial compression. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile–A (60:40) = B. Gradient: 30%(load)–30'–90% B. Flow-rate: 75 ml/min, 33 atm back pressure. Detector: 2.56 (285 nm). Chart speed: 0.5 cm/min.

Preparative purification of hpGRF(1–40)–NH_2

The gradient analysis of crude hpGRF(1–40)–NH_2 was developed in TEAP pH 2.25 on a C_{18} column (Fig. 11) as was the isocratic analysis (Fig. 12). The preparative purification of 2.1 g of the crude peptide was run on a Vydac C_{18}, 17-μm particle size, cartridge in TEAP 2.25–acetonitrile buffer (Fig. 13). A total of 6.3 g of crude hpGRF(1–40)–NH_2 was purified in three runs. Fractions were collected approximately every 75 ml and analysed isocratically in TEAP–acetonitrile buffer (Fig. 14). The fractions from each of the three preparative runs that most resembled fraction 7 in Fig. 14 were then pooled and rerun on a Vydac C_4, 17-μm particle size cartridge in TEAP 2.25–acetonitrile, as were the fractions from each of the three runs that most resembled fraction 8 in Fig. 14, as seen in Fig. 15. The isocratic analyses of the preparative rerun of the fractions shown in Fig. 15 are illustrated in Fig. 16. The fractions from each of the three runs that most resembled fraction 9 in Fig. 14 were then pooled and rerun on the preparative HPLC, as were the three fractions that most resembled fraction 10 in Fig. 13. In this manner, all the impure fractions were processed to a uniform purity with the aid of two different columns, C_{18} and C_4. These fractions, which had a total volume of 3 l after being diluted with water to insure that the peptide adsorbed to the column, were pumped onto the C_4 column

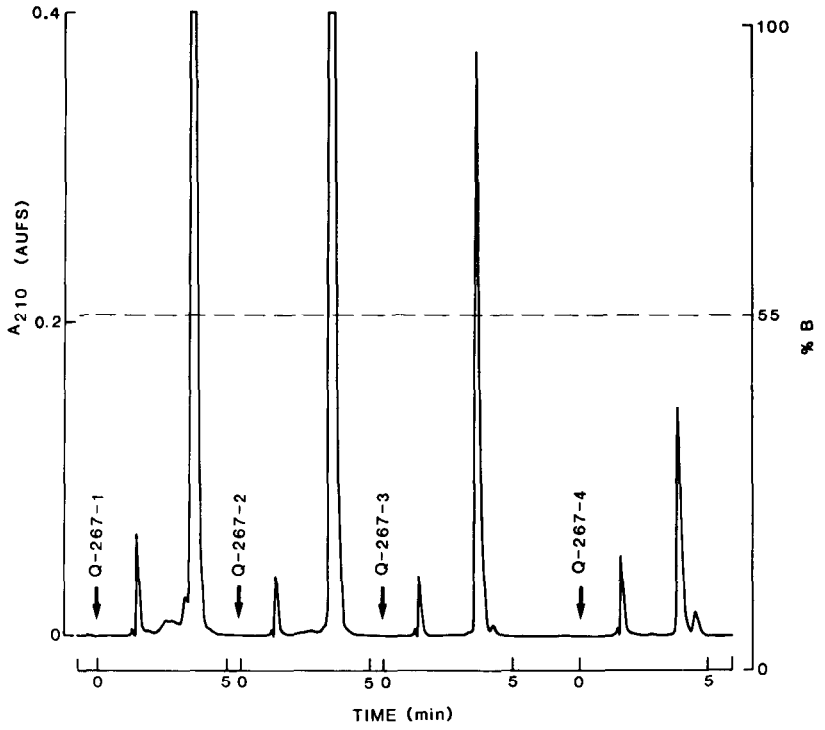


Fig. 9. Load: [Ac-DNal(2)[1],4FDPhe[2],(for)DTrp[3],DArg[6]]-GnRH fractions 1–4, (20 μl, *ca.* 4–20 μg) from preparative desalting shown in Fig. 8. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: TEAP 2.25 = A; acetonitrile–A (60:40) = B. Gradient: 55% B isocratic. Flow-rate: 2.0 ml/min, 2950 p.s.i. Back pressure. Detector: 0.4 (210 nm). Chart speed: 1 cm/min.

and desalted with 0.1% TFA–acetonitrile, (Fig. 17). The three fractions were lyophilized and yielded 60 mg, 345 mg and 137 mg, respectively. The gradient analyses of the three lyophilized fractions (Fig. 18) had purities of 98.0, 99.1 and 94.2%, respectively.

Preparative purification of [DTrp[6],Pro[9]-NEt]-GnRH

Fig. 19 illustrates the analytical gradient analysis of crude [DTrp[6],Pro[9]-NEt]-GnRH in TEAP 2.25–acetonitrile buffer and (Fig. 20) the isocratic analysis of the same peptide. The crude peptide (54 g) was purified 3 g at a time. The excellent reproducibility of the system is illustrated (Fig. 21) by the tenth and seventeenth preparative runs, and their isocratic analysis (Fig. 22). Analytical profiles of fractions of comparable purity from the two selected runs are shown in successive columns arranged by the approximate purity of each cut. The "40% phillics" indicating 40% contamination with hydrophilic impurities were then pooled and rerun preparatively under the same conditions. The fractions were analysed isocratically and the appropriate pools were made. The "15% phillics" were then rerun preparatively in the same buffer, fractions were analysed and combined in the appropriate pools. Finally the "5% phillics" were rerun preparatively, analysed isocratically and pooled (if pure enough) into the ">99%" fraction. Pools of fractions containing hydrophobic im-

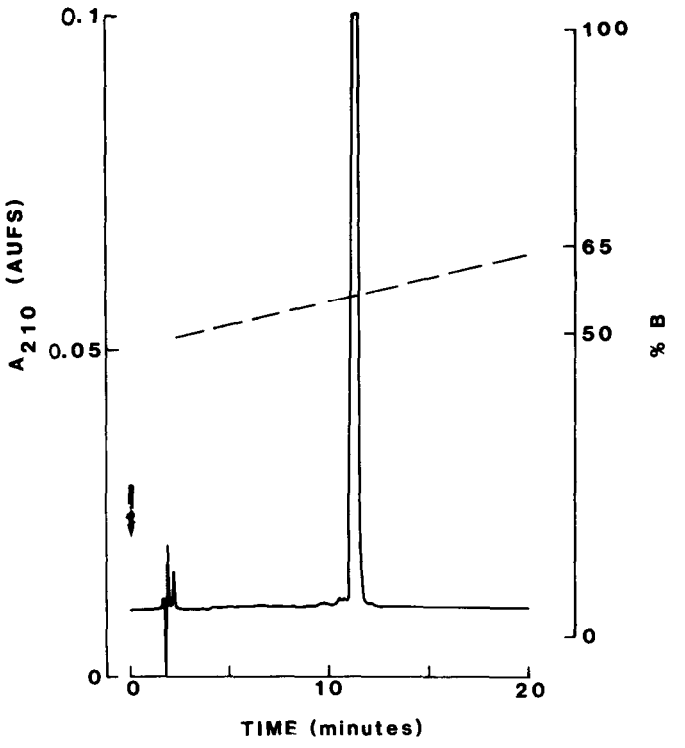


Fig. 10. Load: lyophilized pool of fractions 2 and 3 [Ac-DNal(2)',4FDPhe^2,(for)DTrp^3,DArg^6]-GnRH (20 μl, 20 μg) from preparative desalting shown in Fig. 8 and analysed in Fig. 9. Column: Vydac 5 μm, C_{18}, 25 × 0.46 cm I.D. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile–water (60:40) = B. Gradient: 50%–20'–65% B. Flow-rate: 1.8 ml/min, 3000 p.s.i. back pressure. Detector: 0.1 (210 nm). Chart speed: 0.5 cm/min. Overall yield: 128 mg of 99.7% purity from 1.2 g.

purities were often generated and were processed similarly from the most impure stage independently of the pools containing hydrophilic impurities. Fractions that contained less than 60% pure material or which had too little material to warrant further purification were discarded. Once all fractions had been run to what we considered the point of diminishing return or pooled into the "> 99%" fraction, they were desalted in 0.1% TFA–acetonitrile. Desalting of as much as 5 g of peptide was achieved in one run without any evidence that the total capacity of the column had been reached.

Purification of Boc-N$^{\alpha}$CH$_3$Ser(OBzl) on Vydac underivatized silica (15–20 μm) was attempted using the following conditions: buffer A = chloroform–1% acetic acid; buffer B = chloroform–methanol (1:1); gradient conditions 5 → 60% B in 60 min; flow-rate 100 ml/min. This solvent system, which was in fact too strong, did not achieve any separation. Rather than repeating this experiment with a less polar solvent system we achieved a perfect separation using reversed-phase chromatography (C_{18}:buffer A = 0.1% TFA, buffer B = 60% acetonitrile in A; gradient conditions 40 → 75% in 60 min; flow-rate 100 ml/min). *A posteriori*, a rough calculation of solvent costs for both approaches indicated that reversed-phase chromatography could be advantageously used for such purifications (protected amino acids

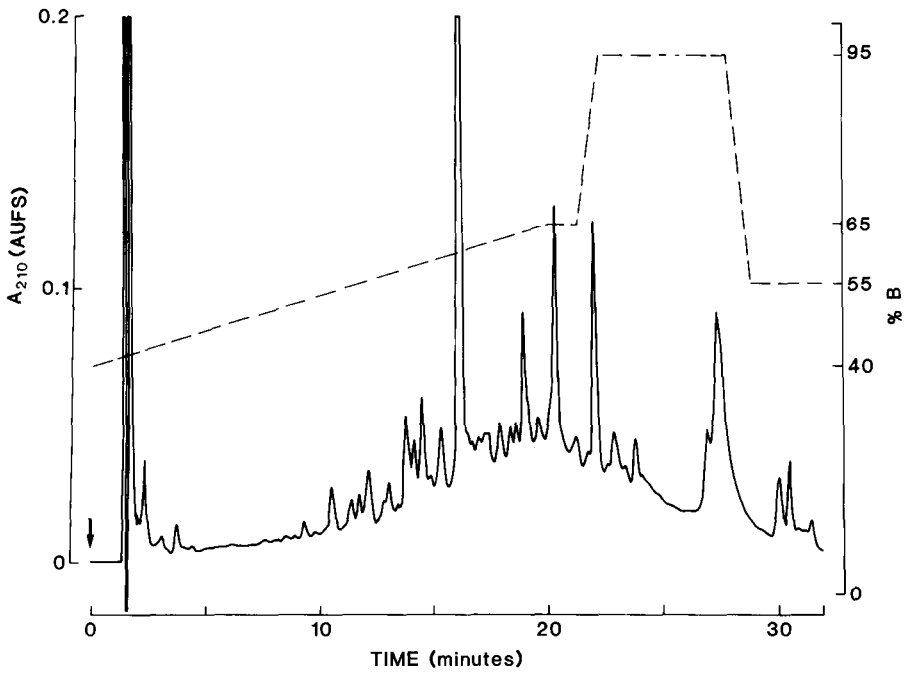


Fig. 11. Load: lyophilized hpGRP(1–40)–NH_2 from HF cleavage (20 μl, *ca.* 20 μg), see Table I for structure. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile–water (60:40) = B. Gradient: 40%–20′–65% (3′)–1′–95% B. Flow-rate: 2.0 ml/min, 3000 p.s.i. back pressure. Detector: 0.2 (210 nm). Chart speed: 1 cm/min.

and peptides) where loading of the normal phase cartridges is limited by the complexity of the mixture to be separated.

RESULTS AND DISCUSSION

The general conditions to be fulfilled by any analytical HPLC system are that it be resolutive, reproducible, accurate and be devoid of artifact (memory effect for example).

In the case of a preparative separation one should add practicality and economy as compared to other processes.

In our earlier paper[11] describing a new series of column supports and two recognized solvent systems[15,16] as applied to the separation of insulins we demonstrated the resolutive power and reliability of prepacked analytical columns from Vydac. Excellent recovery (and concomittant lack of memory effect) obtained on those columns was best illustrated by the isolation of rat hypothalamic CRF[14] and rat[20] and human[26] hypothalamic GRFs.

In order to extrapolate from the analytical to preparative scale we obtained bulk silica identical to that used for analytical columns (with the exception of particle size) from The Separations Group and empty cartridges and frits from Waters Assoc. Then we demonstrated that 10-μm particle size silica for example did not wash through frits which average pore size is 15–25 μm nor plug them as one could have

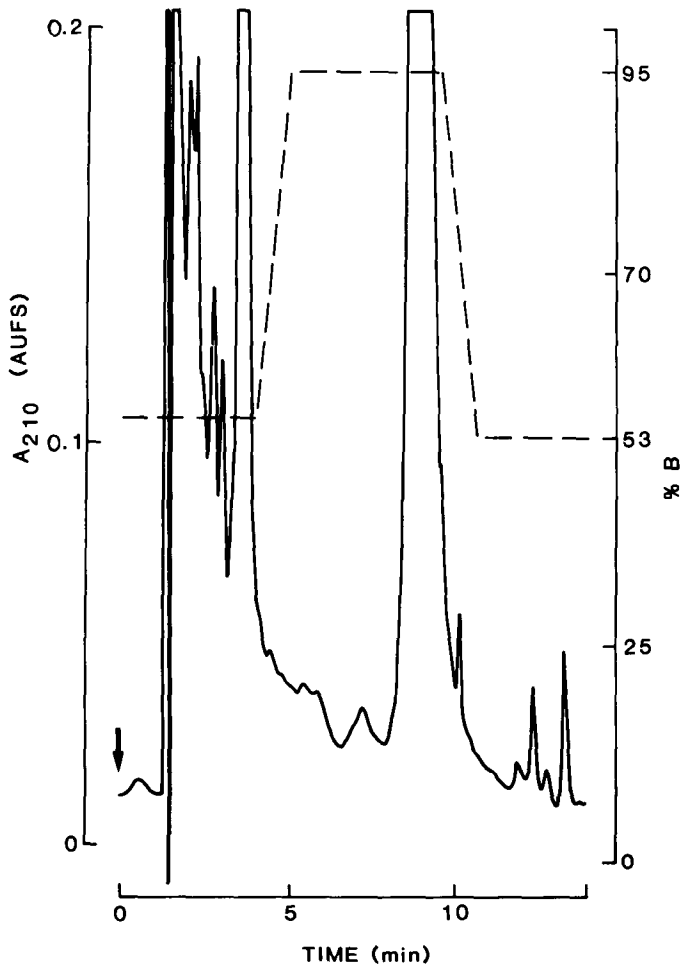


Fig. 12. Load: lyophilized hpGRP(1–40)-NH_2 from HF cleavage (20 μl, *ca.* 20 μg). Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile–water (60:40) = B. Gradient: 53%(4')–1'–95% B. Flow-rate = 2.0 ml/min, 3000 p.s.i. back pressure. Detector: 0.2 (210 nm). Chart speed: 1 cm/min.

expected. To make this approach practical we had to find an easy way to generate accurate gradients (see *Apparatus*) and finally we had to compare costs with other processes available for peptide purification.

Most points have been addressed in the Experimental section and that relating to cost became quickly irrelevant since no other method could be used that would outperform reversed-phase HPLC not only in terms of resolutive power, but also in terms of time involved per separation. The outstanding durability of the Vydac silica supports, the fact that they can be used for more than one biologically active peptide (contrary to what we had observed with the original 125-Å C_{18} cartridges from Waters Assoc.) without cross-contamination if appropriate washing steps are introduced (including extensive rinsing of all lines and pumps if the material is loaded

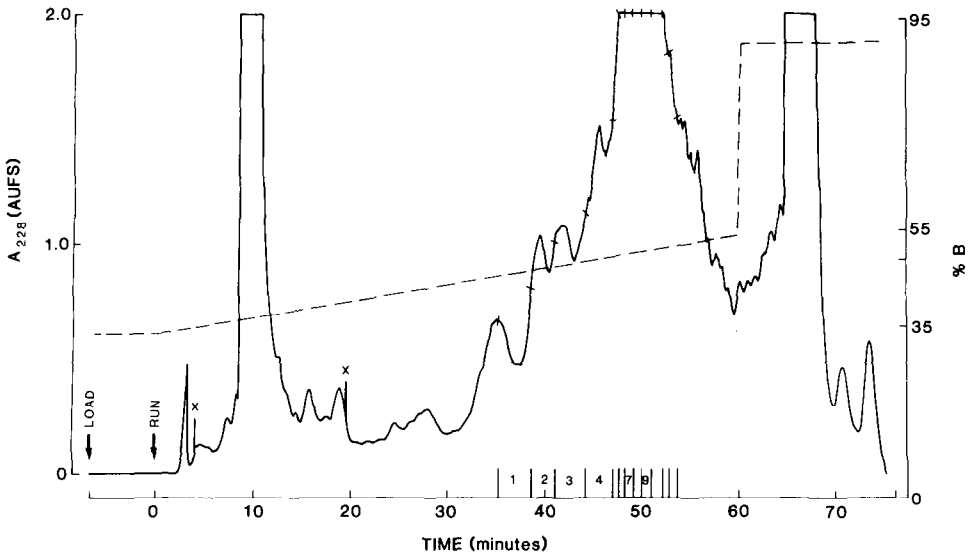


Fig. 13. Load: lyophilized hpGRF(1–40)–NH_2 from HF cleavage (400 ml, 2.1 g). Column: PrepPak 500, 30 × 5 cm I.D., packed with Vydac 17 μm C_{18} material, 40 atm radial compression. Buffers: TEAP 2.25 = A; acetonitrile–A (60:40) = B. Gradient: 35% (load)–60′–55%–1′–95% B. Flow-rate: 100 ml/min, 18 atm back pressure. Detector: 2.0 (228 nm). Chart speed: 0.5 cm/min.

through the pumping system) and that up to six different runs can be performed within an 8–10 h period, easily compensate for solvent and column costs.

In more detail, the strategy developed for the reversed-phase HPLC purification of polypeptides involved the following steps:

(1) Optimization of analytical gradient conditions that show the nature of the peptidic mixture: presence or absence and relative abundance of a major component, its relative hydrophobicity, and extent of contamination by closely related impurities.

(2) Determination of the analytical isocratic conditions for which the desired product would elute at a k' of 2.5–4.5.

(3) Application of the crude peptide mixture (2.0–4.0 g) dissolved in the appropriate buffer (300–500 ml) through the pumps onto the cartridge (possibly after filtration).

(4) Generation of an appropriate gradient of acetonitrile that will achieve the desired separation.

(5) Analysis of each 50–150 ml fraction which was generated.

(6) Pooling of the fractions of comparable purity.

(7) Rerunning separately those fractions containing hydrophilic and/or hydrophobic impurities.

(8) Reapplication after dilution (1:1) with water of all good fractions for desalting.

(9) Generation of a steep gradient in order to obtain the desired product in a small volume unless further purification is desired from that step.

Running a gradient analytically allows for the optimal separation of the peptide from its impurities; developing a gradient that can be used for all the analogues of the compound that are going to be purified allows the purification conditions to

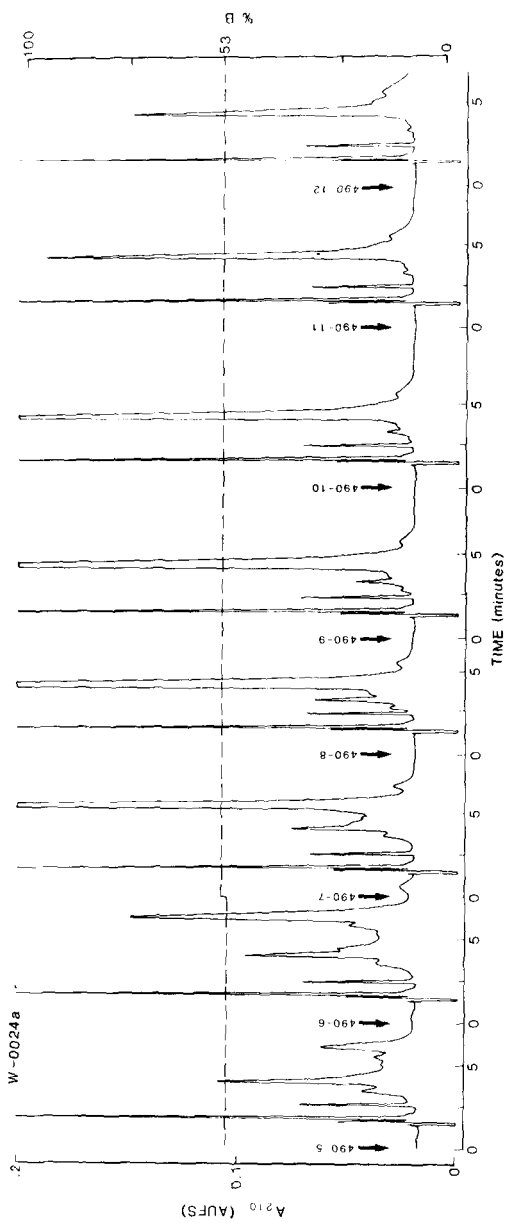


Fig. 14. Load: hpGRF(1–40)-NH_2 fractions 5-12 (20 μl, *ca.* 20 μg) from preparative purification shown in Fig. 13. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile–water (60:40) = B. Gradient: 52.5% and 53% B isocratic, after fraction 6. Flow-rate: 2.0 ml/min, 3000 p.s.i. back pressure. Detector: 0.2 (210 nm). Chart speed: 1 cm/min.

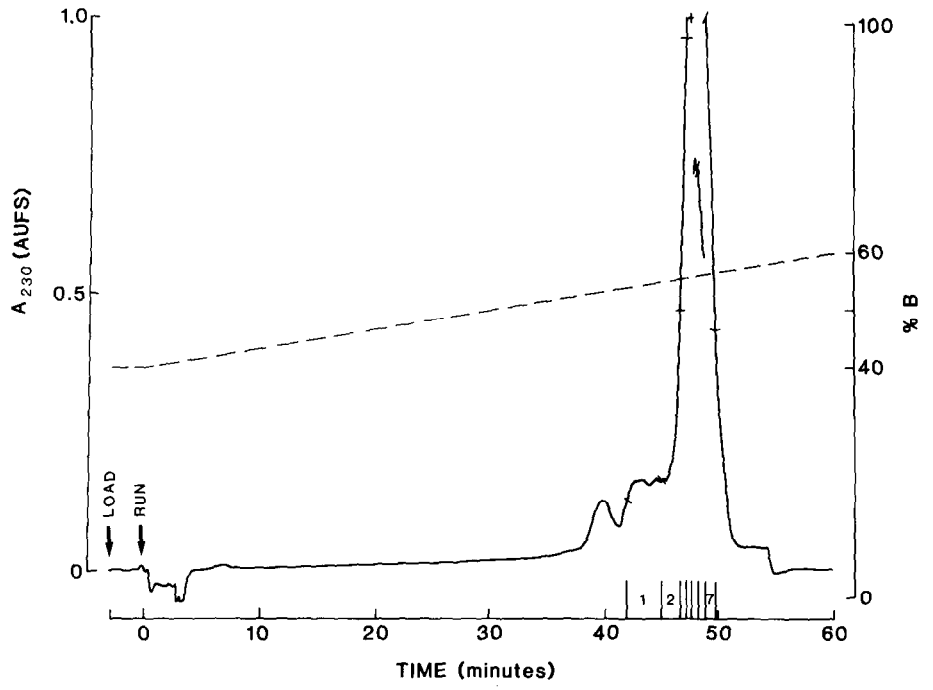


Fig. 15. Load: hpGRF(1–40)–NH_2 3 × fraction 8 (300 ml, *ca.* 0.2 g) from preparative purification shown in Figs. 13 and 14. Column: PrepPak 500, 30 × 5 cm I.D. packed with Vydac 17 μm C_4 material, 40 atm radial compression. Buffers: TEAP 2.25 = A; acetonitrile–A (60:40) = B. Gradient: 37%(load)–60′–57% B. Flow-rate: 100 ml/min, 20 atm back pressure. Detector: 1.0 (230 nm). Chart speed: 0.5 cm/min.

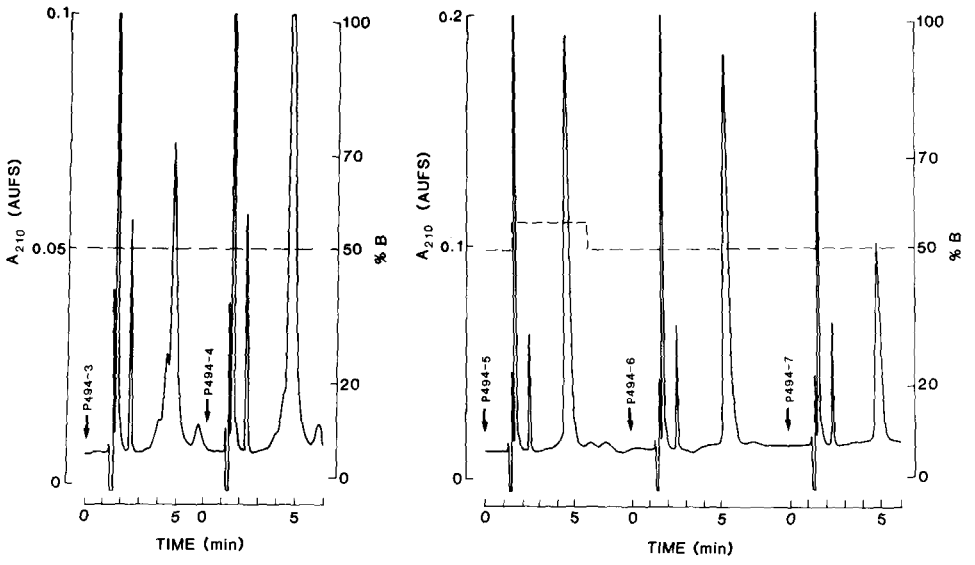


Fig. 16. Load: hpGRF(1–40)–NH_2 fractions 3–7 (20 μl, *ca.* 20 μg) from preparative purification shown in Fig. 15. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile–water (60:40) = B. Gradient: 50% B isocratic; pump problems on fraction 6. Flow-rate: 2.0 ml/min, 3000 p.s.i. back pressure. Detector: 0.1 (210 nm). Chart speed: 1 cm/min.

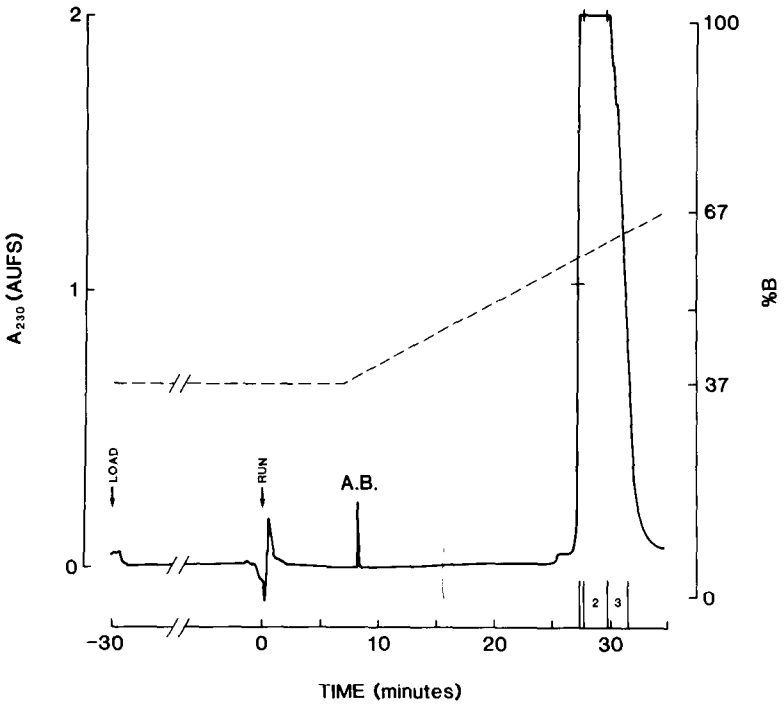


Fig. 17. Load: hpGRF(1–40)–NH_2 fractions from five preparative C_4 purifications (3000 ml, *ca.* 550 mg) shown in Fig. 15. Column: PrepPak 500, 30 × 5 cm I.D., packed with Vydac 17 μm C_4 material, 40 atm radial compression. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile–water (60:40) = B. Gradient: 37%(load + 5')–30'–67% B. Flow-rate: 100 ml/min. 20 atm back pressure. Detector: 2.0 (230 nm). Chart speed: 0.5 cm/min. A.B. = air bubble spike.

be established much more quickly. In Fig. 2, for example, GnRH antagonists which are particularly hydrophobic are routinely chromatographed in TEAP 2.25 buffer from 45% to 95% B over 30 min; the purity of the peptide was assessed and the best conditions for the isocratic and preparative conditions could be empirically determined. The main goal was to optimize the gradient conditions in such a way that appropriate separation was achieved within a minimum amount of time thus also saving solvents. The peptide however should not crash through, hence, increasing the concentration of the B buffer (*i.e.* 100% acetonitrile) by 0.2–0.3% per min at 100 ml/min and starting the gradient a few per cent acetonitrile below the percentage used for the isocratic conditions was found to be reasonable (see Experimental section and figure legends for the three given examples). We have found that gradients of acetonitrile shallower than 0.2% per min at 100 ml/min resulted in peak broadening with little improvement on overall separation or recovery. In general, however, the more hydrophilic the peptide, the more shallow the gradient could be before peak broadening upset resolution. Conversely, in the same system, an upper limit of 1% acetonitrile per min was the point at which sharp peaks started overlapping.

The isocratic conditions (Figs. 3, 12 and 20) have to be determined in order to allow for the successive and rapid analysis of the preparative fractions before the preparative HPLC column was washed and equilibrated for the next run. Most

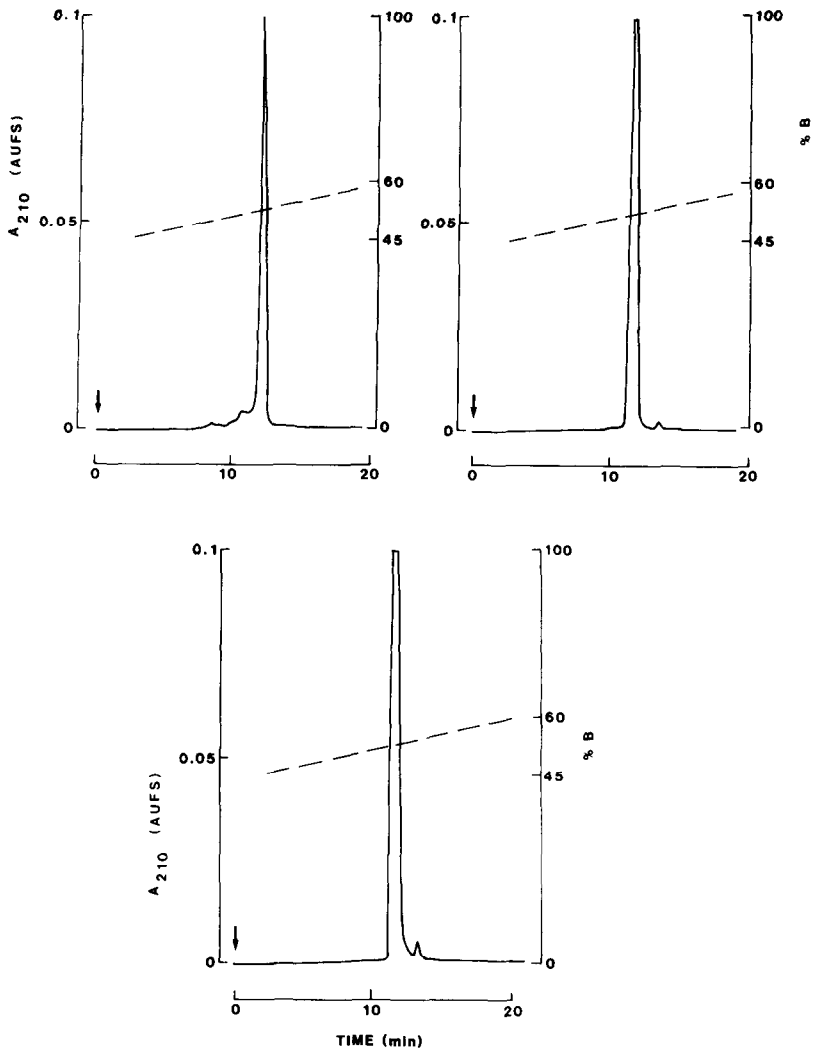


Fig. 18. Load: hpGRF(1–40)-NH_2 fractions 1–3 (20 μl, *ca.* 20 μg) from preparative desalting shown in Fig. 17. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: 0.1% TFA in water = A; 0.1% TFA in acetonitrile–water (60:40) = B. Gradient: 45%–20′–60% B. Flow-rate: 1.8 ml/min, 2800 p.s.i. back pressure. Detector: 0.1 (210 nm). Chart speed: 0.5 cm/min. Overall yield: 60 mg of 97%, 345 mg of 99.1% and 137 mg of 94.2% pure compound from 6.3 g crude.

closely related impurities revealed in the gradient analysis could be separated in 4–6 min or at least shown to be present. Whereas the isocratic analysis of the crude peptide required that the column be submitted to a wash cycle (95% B), this was not necessary anymore for the analysis of the fractions generated by the preparative run since they only contained very closely related impurities which would elute under the chosen isocratic conditions.

Because we had found it more convenient to work with one cartridge and two solvent systems to maximize selectivity rather than that with one solvent and two

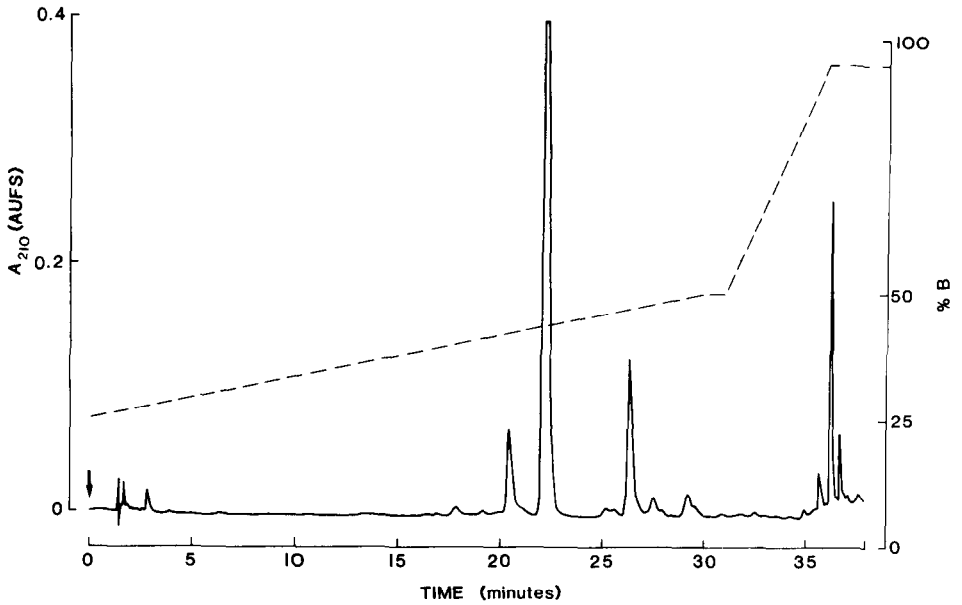


Fig. 19. Load: lyophilized [DTrp^6,Pro^9-NEt]-GnRH from HF cleavage (20 μl, *ca.* 20 μg). Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: TEAP 2.25 = A; acetonitrile-A (60:40) = B. Gradient: 30%(2')-25'-40%(1')-3'-95% B. Flow-rate: 2 ml/min, 3000 p.s.i. back pressure. Detector: 0.4 (210 nm). Chart speed: 0.5 cm/min.

different cartridges we used a C_{18} cartridge and the TEAP 2.25–acetonitrile buffer system first followed by the volatile 0.1% TFA–acetonitrile which allowed for direct lyophilization of the generated fractions. (See purification of [Ac-DNal(2)^1,4FDPhe^2,(for)DTrp^3,DArg^6]-GnRH and [DTrp^6,Pro^9-NEt]-GnRH.)

Once the peptide was eluting from the column, fractions were collected every 50–100 ml and analysed isocratically. The column was then washed and equilibrated as illustrated (Figs. 4, 13 and 21) for the next run. (Overall duration of such a run was rarely greater than 90 min.)

Examination of the isocratic analysis of the fractions generated preparatively often showed hydrophilic UV absorbing entities that appeared proportional to the volume injected and were suspected to originate from the unfiltered TEAP 2.25 buffer. The peak at 2.5 min (Fig. 5) for example was of constant height throughout the analysed fractions.

Unusual observations may result from different selectivities of the analytical and preparative columns used. In Fig. 5, fraction 7, there was an analytically hydrophobic impurity at 4.4 min, that decreased as the more preparatively hydrophobic fractions 8 and 9 were analysed, *i.e.*, this impurity peak reached its maximum concentration before the desired peptide itself reached its maximum concentration (in fraction 8) behaving as if it eluted hydrophilically to the main peak on the preparative column. As expected however the small hydrophobic shoulder in fraction 11 and peak in fraction 12 (retention time 4.2 min) were shown to be the preparative hydrophobic peak centered in fraction 13 (Fig. 4).

The exact reason for this common difference in selectivity has not been fully

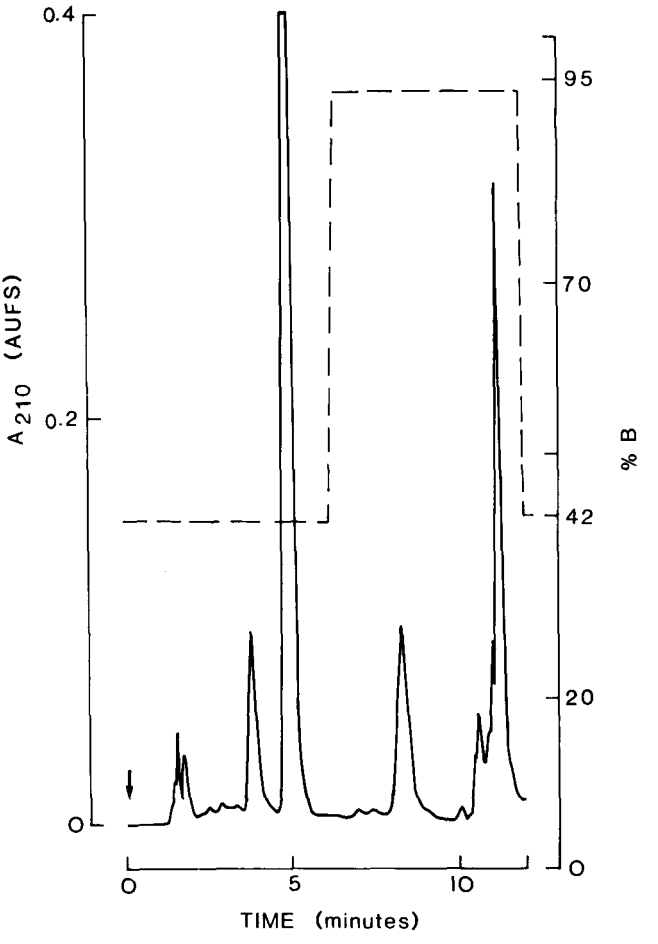


Fig. 20. Load: [DTrp^6,Pro^9-NEt]-GnRH from HF cleavage (20 μl, *ca.* 20 μg). Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: TEAP 2.25 = A; acetonitrile–A (60:40) = B. Gradient: 42%(5')–0.1'–95% B. Flow-rate: 2 ml/min, 3000 p.s.i. back pressure. Detector: 0.4 (210 nm). Chart speed: 1 cm/min.

explored, but may be due to such variances in the analytical and preparative columns as caused by differences in endcapping or the use of rigid (metal) *versus* malleable (plastic) walls.

For desalting purpose, the desired peptide fractions from an earlier step were diluted by an equal volume of deionized water and loaded onto a column equilibrated with 0.1% TFA buffer. Depending on whether one wanted to take advantage of the differences in selectivity offered by a different solvent system (see Fig. 6, desalting [Ac-DNal(2)^1,4FDPhe^2,(for)DTrp^3,DArg^6]-GnRH) or whether one wanted to obtain a peptide fraction as concentrated as possible, one would design a desalting step that would be of different steepness (see Figs. 6, 8 and 17). The trade-off between optimal separation and peak broadness has been discussed earlier. We have shown a difference in selectivity in changing from TEAP 2.25 to 0.1% TFA. Similarly a difference in selectivity in changing from C_{18} to C_4 is illustrated (Figs. 13 and 15).

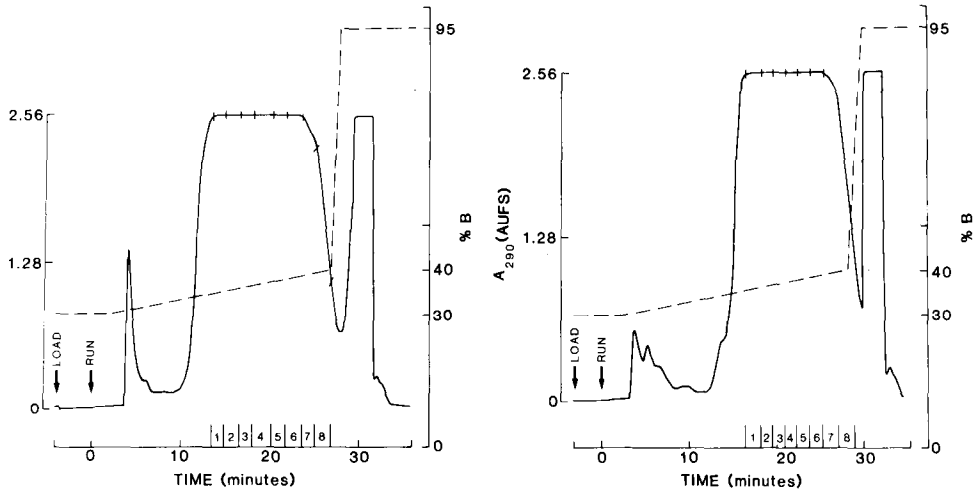


Fig. 21. Load: [DTrp^6,Pro^9-NEt]-GnRH from HF cleavage, runs no. 10 and 17 from eighteen identical runs (300 ml = 3.0 g). Column: PrepPak 500, 30 × 5 cm I.D., packed with Vydac 15–20 μm C_{18} material, 40 atm radial compression. Buffers: TEAP 2.25 = A; acetonitrile–A (60:40) = B. Gradient: 30%(2')–40'–40%–1'–95% B. Flow-rate: 125 ml/min. 25 atm back pressure. Detector: 1.056 (289 nm). Chart speed: 0.5 cm/min.

Not described here is the selectivity that can be obtained preparatively when using cartridges filled with diphenyl derivatized silicas; similar observations to those reported earlier[11] could be made (*i.e.* tighter binding of phenylalanine or tryptophan containing peptides as compared to their behaviour on C_{18} for example).

Loading peptides in large volumes and obtaining sharp peaks, generally posed no problem if the loading buffer strength was adequate; as much as 5 g of peptides diluted in as many as 10 l were routinely desalted in one run. One should remember that overloaded conditions can only be used at the expanse of resolution. In Fig. 21, the column was obviously overloaded but because the "close" impurities were more hydrophilic than the desired product and the runs were consistant, the column could be run in an overloaded condition of 3 g per run. Another advantage of run consistency was that it allowed for optimized fractionation. In Fig. 21, for example, the amount of material collected as >99% pure was increased from *ca.* 1/2 in run 10 to *ca.* 2/3 in run 17 (*cf.* Fig. 22 for the analyses). Especially note the drastic change in going from fraction no. 343-2, 15% hydrophilic impurities, to fraction no. 343-3, 99% pure.

We have found that hydrophilic impurities were easier to eliminate than hydrophobic ones; whether this may be due to a certain amount of peak tailing encountered in the purification of peptides on reversed-phase packing or whether other factors may be involved was not investigated. It resulted however in poorer recovery of the main product when removing hydrophobic impurities riding the tail of the main peak than when eliminating a hydrophilic impurity from its sharp front.

As part of the peptide sequences chromatographed using this technique we can find all naturally occurring amino acids as well as a great number of unnatural or D-amino acids, indicating that no amino acid can be singled out as interfering with the chromatographic process. We have shown however that some highly constrained

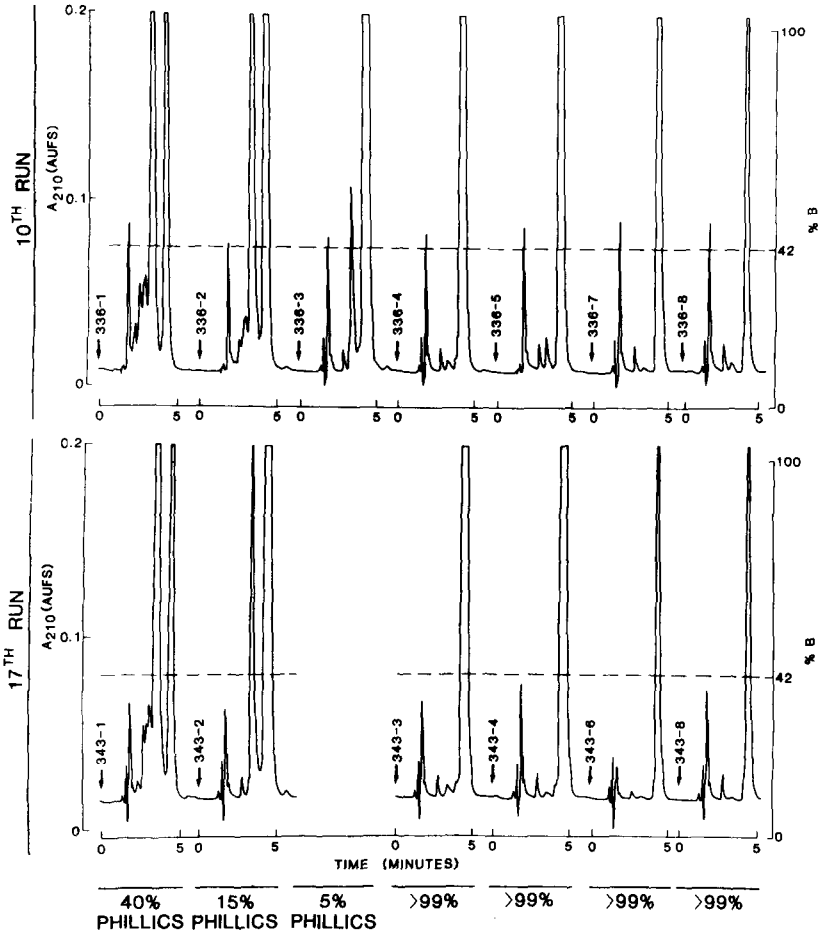


Fig. 22. Load: [DTrp^6-Pro^9-NEt]-GnRH fractions 1–8 (20 μl, *ca.* 20 μg) from the tenth and seventeenth preparative purifications shown in Fig. 21. Column: Vydac 5 μm C_{18}, 25 × 0.46 cm I.D. Buffers: TEAP 2.25 = A; acetonitrile–A (60:40) = B. Gradient: 42% B isocratic. Flow-rate: 2 ml/min, 3000 p.s.i. back pressure. Detector: 0.2 (210 nm). Chart speed: 1 cm/min.

structures such as that of the conotoxin M1 may lead to unusual chromatographic patterns which could be explained by the presence of two conformers in equilibrium at room temperature[24]. It is evident that such behaviour at a preparative scale may also be a problem if not recognized.

In summary, in order to decide to use this purification method exclusively in our laboratory we had to examine thoroughly its potential as well as the economic and safety aspects (since large amounts of acetonitrile are being used). It became quickly evident that we could obtain on the preparative cartridges the selectivity which we had observed with the analytical columns using different solvent systems, therefore we had the needed armamentarium that would allow us to solve most of our separation problems. The safety aspect was also investigated and acetonitrile levels were monitored in the room (15 × 17 ft.) that housed two analytical and two

preparative machines and found to be significantly below the acceptable levels as defined by OSHA.

In terms of the economics of the technique it was found that reagent grade acetonitrile bought in bulk could be used for the usual purification while distilled acetonitrile should be used for the desalting step. There being no alternative technique (except possibly for low pressure reversed-phase chromatography) for the adequate purification of peptides the size of the newly discovered growth hormone releasing factors or corticotropin releasing factors, we have been using this method exclusively. Another approach consisting of improving the quality of our crude peptide mixture is under investigation and should allow us further to extend the size limit of available synthetic peptides which, thanks to such HPLC techniques as the one reported here, is presently in the range of 40–50 residues long.

In conclusion, by means of repetitive manipulations we have been able to isolate large amounts of highly purified peptides in good yield (batches of up to 25 g of final product). An ion-exchange step to obtain the acetate salt or elution from the reversed-phase columns with 0.05 *N* HCl–acetonitrile to obtain the HCl salt, rather than the trifluoroacetate salt, can be performed in order to obtain peptides to be used for human investigation. We are presently investigating the possibility, using considerably larger pressurized columns, of proceeding from the preparative to the industrial scale.

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