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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF PEPTIDES

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SUMMARY

Peptides varying in size from di- to decapeptide have been subjected to high-pressure liquid chromatography on Phenyl-Corasil, Poragel PN, and Poragel PS under reversed-phase conditions with acetonitrile–water mixtures. It has been found that residual silanol groups in the Phenyl-Corasil and the functional groups in the Poragels significantly influence retention. Peptides were also chromatographed on a Hydrogel IV gel filtration packing with aqueous eluants, and it was found that effects other than gel filtration play a major role in determining retention.

INTRODUCTION

Merrifield's "solid phase" peptide synthesis and the subsequent automation has proven to be a valuable tool for the peptide chemist. A large number of analogs of physiologically active peptides may now be synthesized by this method considerably faster than by "classical" solution chemistry. However, in the absence of 100% yield in each coupling and deprotection step, purification of the final product can be a major problem, as many of the impurities will be truncated and deletion sequences with properties similar to the main product will occur. There is need for chromatographic procedures which can separate closely related peptides, and high-pressure liquid chromatography (HPLC) with its generally high performance should be applicable.

Isolation of peptide hormones from the hypothalamic tissue of the brain is another field of research where high-performance chromatographic procedures are essential. Often a very small amount of the peptide hormone is present in early fractions. The desire for high sensitivity in a non-destructive detection should make HPLC particularly suitable for isolation of natural hormones.

Due to the polar nature of unprotected peptides, they are usually not purified by adsorption chromatography, and chromatography by gel filtration, partition, and ion-exchange methods have been used extensively in gravity LC along with countercurrent distribution and electrophoresis. The packing materials specifically prepared for HPLC have been used very sparsely, and only a few reports of peptide separation on HPLC packings are found in the literature.

Reversed-phase chromatography on C_{18}-Corasil has been used for purification and identification of isariin and linear derivatives[1]. Analysis of bacitracin has been performed on C_{18}-Corasil[2], but better resolution was obtained on μBondapak C_{18} (ref. 3). This microparticulate packing material was also used for the reversed-phase separation of N-phenyl-thiocarbamyl-peptides[4]. Three N-protected dipeptide esters were separated on silica by normal adsorption chromatography[5]. Ion exchange of peptides, utilizing different cation exchangers developed for automatic amino acid analyzers, and applicable within a limited pressure in HPLC, has been more frequently described[6,7]. In one case of cation exchange, it was found[6] that gel filtration effects contributed significantly to the retention. The use of HPLC in purification of hypothalamic factors of supposedly peptidic nature on Phenyl-Corasil and different Poragels has been reported[8,9]. The purpose of this study was to gain knowledge about peptide behavior on different HPLC packings and to evaluate their use in analysis, purification, and isolation of peptides of synthetic and natural origin.

EXPERIMENTAL

The peptides were synthesized* with protected L-amino acids by a solid phase technique on a Beckman Model 990 automatic peptide synthesizer, except for <Glu–His–OH and <Glu–His–Trp–OH, which were synthesized by classical solution methods[10]. After deprotection, they were purified by conventional gravity chromatography, until they proved TLC homogenous and the amino acid analytical data were satisfactory.

The HPLC apparatus used was either a Waters Type ALC 100 instrument equipped with a 1000-p.s.i. or a 3000-p.s.i. reciprocating pump or a Waters Type ALC 200 instrument furnished with a Model 6000 solvent delivery system. Detection was done by UV adsorption, either by using the standard Waters 254 nm UV detector or by using this detector series-connected after a Beckman/Waters LC-25 UV detector with an 18-μl flow cell operating at 220 nm.

Solvents were doubly distilled deionized water and acetronitrile (UV grade, distilled in glass, Burdick and Jackson Labs., Nustelgon, Mich., U.S.A.). The solvents were mixed by vol.-% and allowed to equilibrate at room temperature. No degassing operations were performed.

The HPLC packing materials used were Bondapak Phenyl-Corasil, Poragel PN (37–75 μm), Poragel PS (37–75 μm), and Hydrogel IV (37–75 μm) from Waters Assoc. (Milford, Mass., U.S.A.). In all cases 3 ft × 1/8 in. stainless-steel columns with end fittings containing 5-μm sintered metal filters were slowly dry packed by adding small amounts of packing material followed by tapping and vibrating after each addition. The amount of packing material in a 3 ft. × 1/8 in. column was 5.7 g for Phenyl-Corasil and *ca.* 2 g for the Poragels.

All chromatographic runs were performed on a 3 ft. × 1/8 in. column with isocratic elution at ambient temperature. Flow rate was 1.0 ml/min with a pressure drop over the column generally ≤ 1000 p.s.i. Samples were introduced with a microsyringe through a septum injector during flow. Generally, the amount of peptide injected was 50 μg dissolved in 5–20 μl water.

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* Details of the solid phase synthesis will be published separately, but see for example ref. 10.

The chromatographic peaks were identified by their UV characteristics, and also by amino acid analysis after preparative runs.

RESULTS

The retention of a component on a column can be expressed as elution time, elution volume (V_e), reduced elution volume ($\equiv V_e - V_0$), and R_F ($\equiv V_0/V_e$). In HPLC, the capacity factor (k') is mainly used:

$$k' = \frac{V_e - V_0}{V_0}$$

where V_0 is the column void volume or the elution volume of an unretained sample.

For the determination of V_0, when the peptide is retained, small impurities or solvent peaks are seen very early in the chromatogram, and these elute without retention. After correction (if necessary) for the tubing dead volume, it was found fairly consistently that $V_0 \approx 1.7$ ml for these 3 ft. × 1/8 in. columns, both for the superficially porous Phenyl-Corasil and for the totally porous organic polymers (Poragels and Hydrogel). V_0 seemed to be independent of the solvent. The columns, after solvation with either water or 70% acetonitrile, showed no visible shrinking or swelling of the packing materials.

Phenyl-Corasil

Phenyl-Corasil is like the C_{18}-packings, which are regarded as reversed-phase packing. It is normally eluted with polar solvents, which elute first the most polar components. An increase in solvent polarity gives an increase in sample retention.

Table I shows the capacity factors for various peptides on Phenyl-Corasil

TABLE I

k' VALUES OF PEPTIDES ON PHENYL CORASIL

Peptide	*k'*				
	H_2O	*10% CH_3CN*	*20% CH_3CN*	*40% CH_3CN*	*70% CH_3CN*
<Glu–His–OH	0.3			0	
<Glu–Ser–Gly–OH	0	0		0	0
<Glu–Ser–Asp–OH	0				
<Glu–His–Trp–OH	4.7	0.9	0.4	0.1	
<Glu–His–Pro–Gly–OH	1.6	0.2	0.2	~ 0	
<Glu–His–Trp–Lys–Tyr–Pro–OH	>15		≈15	3.4	1.3*
<Glu–His–Tyr–Trp–Lys–Pro–OH	>15		>20	6.8	1.2*
<Glu–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–OH	>10	2.8	0.3	~ 0	0.2
<Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 (LHRH)			>20	>20	>20

* 100 μg peptide in 20 μl 70% CH_3CN was injected. Both the effects of decreasing sample load and water as injection solvent will increase retention.

eluted with water and with different acetonitrile–water mixtures. The data confirm the reversed-phase behaviour under these conditions, as water is a less effective eluant than acetonitrile–water mixtures. The relative polarity of the peptides is more difficult to estimate. However, lengthening of <Glu–His– with –Pro–Gly– or –Trp– would be expected to decrease the peptide polarity, and an increase in retention is observed, which again confirms a reversed-phase behavior. Table I shows capacity factors expressed as > 10, 15, or 20, which means that either no peaks were observed within the corresponding elution volume, or if peaks were observed, they accounted for a small proportion of the injected sample. This latter situation is exemplified by the decapeptide LHRH. Elution with 70% acetonitrile shows a rather large early peak, but a preparative (10 mg) run on a 6 ft. × 3/8 in. Phenyl-Corasil column gave a weight recovery of only 7%. Most of the peptide was retained on the column.

The mechanism of retention in reversed-phase chromatography has been a matter of concern. Liquid–liquid partition between the polar mobile phase and a less polar stationary phase formed by interaction between the eluant and the bonded phase has been suggested[11]. It was also considered that retention is due to a non-polar adsorption[12]. There seems little doubt that the actual retention mechanism is a complex phenomenon including both of these two effects, and is dependent on the type of packing material and other chromatographic conditions.

In reversed-phase chromatography, it is usually assumed that polar adsorption to residual silanol groups on the silica support has a minor role. However, this is not necessarily correct for peptides on Phenyl-Corasil. The results in Table I indicate that a peptide is more retained with the more basic functions it contains, and this retention is presumably due to polar interactions between the basic groups and residual acidic silanol groups of the silica support. This effect is also seen from the retention of different amino acids on Phenyl-Corasil in water: Glu, k' = approx. 0; Gly, 0; Tyr, 0; Phe, 0.2; Trp, 0.2; His, 2.4; Lys, > 20; Arg, > 20.

The polar amino acids Gly and Tyr elute in front together with the acidic Glu followed closely by the relatively less polar Phe and Trp. Later, the weakly basic His elutes. The stronger basic Lys and Arg are even more retained. By injection of ng amounts of the aromatic amino acids, they can be separated in the elution order Tyr, Phe, and Trp. This order, followed much later by His, was observed[13] during elution of these amino acids in water on Corasil II by adsorption chromatography. By comparison of the k' values given in Table I and above it is seen that <Glu–His–OH is less retained than His. Another indication of the occurrence of silica adsorption in chromatography of peptides on these reversed-phase packings is the observation[2] that the elution order of bacitracin analogs on C_{18}-Corasil is similar to that obtained by CM-C cation exchange, again showing that basic peptides are more retained.

Two of the main factors which concern the general performance of an HPLC column are selectivity (defined[14] as the capacity of the column to retain different solutes for different times) and the efficiency (the capacity of the column to give narrow solute bands and therefore effect separation in spite of an eventual low selectivity).

Table I shows that Phenyl-Corasil gave good selectivity. It utilizes most of the solvent range, and some peptides did not even elute with 70% acetonitrile while others were not even retained in water. Table I shows that in 20% and in 40% acetonitrile the two hexapeptides of identical amino acid composition but different sequence can be distinguished.

The column efficiency is expressed as the number of theoretical plates of a column (N) or the height of such a plate (H)

$$N = 16\left(\frac{V_e}{W}\right)^2; H = \frac{L}{N}$$

where W is the band width of a solute peak and L is the column length.

For medium retained peaks, *i.e.*, k' between 1 and 5 in Table I, it was found that $N \approx 100$ for the 3 ft. × 1/8 in. Phenyl-Corasil column, corresponding to a plate height of $H \approx 9$ mm. This plate height is in the high range of the H values between 0.05 and 5 mm typically observed in HPLC[15], and it is especially high in comparison to the minimum plate count of 1350/m, corresponding to $H \approx 0.7$ mm, listed by Waters Assoc.[16] for this packing material. In order to determine whether this low efficiency was due to an unsatisfactory packing procedure, a pre-packed Phenyl-Corasil column was obtained from Waters Assoc., and the same low efficiency was observed on this prepacked column.

Another aspect of peptide chromatography on Phenyl-Corasil is the peak tailing often encountered for more retained solutes. <Glu–His–Pro–Gly–OH, eluted in water, gave a relatively little tailing. The peak asymmetry, as defined in ref. 17, was calculated to be 2.0. Associated with the peak tailing was the finding that an increase in the amount of peptide applied on the column caused a decrease in retention. This effect is seen from capacity factors for different sample loads of <Glu–His–Pro–Gly–OH on a 3 ft. × 1/8 in. Phenyl-Corasil column eluted with water: for 200 μg, $k' =$ 1.2; 50 μg, 1.6; 10 μg, 2.3; 5 μg, 2.6; 1 μg, 2.8. This peak tailing and dependency of retention upon sample load indicates a Langmuir-type isotherm and is presumably, at least partially, due to the adsorption effect from residual silanol groups.

The detection sensitivity of an HPLC system is dependent on such factors as k', H, and the mode of detection. For maximum sensitivity, sharp symmetrical peaks with low values of k' and H are preferred. A refractive index detector showed unsatisfactory sensitivity for peptides, so detection was by UV absorption which, in most cases, was monitored both at 254 nm and at 220 nm. Only peptides containing aromatic amino acids have appreciable UV absorption at 254 nm, making the standard 254-nm UV detector unsuitable for peptides containing only aliphatic amino acids. However, the peptide bond has an absorption maximum around 190 nm[18,19] and detection at 210–220 nm will monitor at the base of this absorption peak, thereby making detection at 220 nm both more general for all peptides and more sensitive because of the stronger absorption. The superiority of 220 nm detection is shown for <Glu–His–Pro–Gly–OH, which was eluted with 10% acetonitrile. The minimum amount that gave a detectable peak was *ca.* 50 μg for the 254-nm detector and *ca.* 0.1 μg for detection with the LC-25 at 220 nm. Another drawback of the 254 nm is that small amounts of aromatic impurities can be found as large peaks and actually be the dominant peak in the 254-nm chromatogram, but in the 220-nm chromatogram they were insignificant as compared to the peptide peak.

Poragel PN

The Poragels are cross-linked organic polymers prepared by copolymerisation of styrene with different vinyl components. A similar type of a polystyrene packing

material was investigated[20] for separation of derivatives of aromatic acids and amines, and was found to show reversed-phase behaviour during elution with methanol. Poragel PN also contains bound ester groups[16] which are partially hydrolyzed to carboxy groups, and the presence of this polar functionality might be expected to influence the chromatographic behaviour.

Table II shows the retention of different peptides on Poragel PN eluted with water and acetonitrile–water mixtures. Poragel PN does function in a reversed-phase manner, as water is a less powerful eluant than acetonitrile–water mixtures. Similar to Phenyl-Corasil, small acidic peptides showed little retention, and larger peptides containing basic amino acids were more retained. Presumably, this is due to the presence of free carboxy groups on the polymer giving rise to polar interactions. The behaviour of the peptides on Poragel PN was very similar to their behavior on Phenyl-Corasil with respect to selectivity, retention, and efficiency. However, Poragel PN behaved differently from Phenyl-Corasil in one respect. Phenyl-Corasil generally showed tailing peaks where the retention decreases with increasing sample load. Poragel PN, in contrast, showed peaks with less pronounced fronting[15], and the retention was not so dependent on sample size. When such dependency was observed, there was a slight increase in retention with an increase in sample load.

TABLE II

k' VALUES OF PEPTIDES ON PORAGEL PN

Peptide	*k'*				
	H_2O	*10% CH_3CN*	*20% CH_3CN*	*40% CH_3CN*	*70% CH_3CN*
<Glu–His–OH	0.9			0	
<Glu–Ser–Gly–OH	0.5		≈ 0	0	0
<Glu–Ser–Asp–OH	0.3			0	
<Glu–His–Trp–OH	>15	4.2	0.6	≈ 0	
<Glu–His–Pro–Gly–OH	1.7		0	0	
<Glu–His–Tyr–Trp–Lys–Pro–OH	>20			0.3	0.5
<Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 (LHRH)				≈10	>10

Poragel PS

Like Poragel PN this is a polystyrene-type organic polymer. However, Poragel PS contains pyridine rings as functional groups[16], and this change in functionality is found to affect the chromatographic behavior.

Table III shows that Poragel PS shows reversed-phase character, as decreasing the polarity of the eluant causes a decrease in retention. The increase in retention occasionally seen between 40 and 70% acetonitrile is probably due to limited solubility in the latter solvent. However, the small acidic peptides that eluted essentially unretained on Phenyl-Corasil and Poragel PN are now very retained, and the larger peptides containing basic amino acids elute earlier. This reversal in elution pattern

TABLE III

k' VALUES OF PEPTIDES ON PORAGEL PS

Peptide	*k'*				
	H_2O	*10% CH_3CN*	*20% CH_3CN*	*40% CH_3CN*	*70% CH_3CN*
<Glu-Ser-Gly-OH	>20			⩾10	>15
<Gly-Ser-Asp-OH				>20	>15
<Glu-His-Pro-Gly-OH			7.1	1.2	4.4
<Glu-Ser-Tyr-Gly-Leu- -Arg-Pro-Gly-OH			2.3	0.1	0.1
<Glu-His-Tyr-Ser-Trp- -Gly-Leu-Arg-Pro-Gly-OH			>20	~ 0	0
<Glu-His-Trp-Ser-Tyr- -Gly-Leu-Arg-Pro-Gly- NH_2 (LHRH)	>20			0.2	2.1

corresponding to the change from acidic to basic functional groups emphasizes the influence of polar interactions in these reversed-phase packings.

The peak shape obtained on Poragel PS is symmetrical in contrast to the tailing and fronting peaks obtained on Phenyl-Corasil and Poragel PN, and retention time on Poragel PS shows little if any dependency upon sample load. The peaks appear very broad, showing extremely low efficiency, and a theoretical plate number of 10 to 40 is generally found for the 3 ft. × 1/8 in. Poragel PS column. This corresponds to a plate height between 2 and 9 cm.

Hydrogel IV

Gel filtration chromatography has a number of advantages. Retention is relatively little dependent on eluant, and all components elute in a volume range between the interstitial volume and the total solvent volume of the column in the absence of other than gel filtration effects. Retention is mainly governed by molecular size, roughly equivalent to molecular weight (MW), and the relative polarity of the different components has little influence. Gel filtration packing materials that do not collapse under the pressure applied in HPLC are useful such as the Hydrogel series based on a polar organic polymer compatible with aqueous solvents.

The retention of four different peptides under elution with water and with 40% acetonitrile in water is given in Table IV. Although capacity factors are usually not used for describing retention in gel filtration, they are used here for comparison with the other columns. The total solvent volume of the column is the sum of the interstitial volume and the solvent volume inside the beads, and it is equivalent to what is termed void volume in other chromatographic modes. The elution volume of an unretained solute, which in gel filtratio n is identical to the interstitial volume, has been used for the calculation of *k'* and is taken to be $V_0' \simeq 1.7$ ml for this 3 ft. × 1/8 in. column.

Hydrogel IV is designed for a MW range of *ca.* 500 to 40,000 (ref. 16), so all the applied peptides should be well retained and elute slightly before or in the total solvent volume of the column. This situation seems to be best approached by 40% acetonitrile, but it is evident from Table IV that factors other than gel filtration play a significant role in determining retention. The solvent exerts a major influence on elu-

TABLE IV

k' VALUES OF PEPTIDES ON HYDROGEL IV

Peptide	*MW*	*k'*	
		H_2O	40% CH_3CN
<Glu–Ser–Gly–OH	273	0.3	1.3
<Glu–His–Trp–OH	452	>10	1.7
<Glu–His–Pro–Gly–OH	420	0.9	1.3
<Glu–His–Tyr–Trp–Lys–Pro–OH	840	>20	7.5

tion for at least two of the four peptides, and 40% acetonitrile is a stronger eluant than water in both these two cases, indicating some degree of reversed-phase character. The excessive retention found in these two cases, and the very broad peaks generally obtained also show the influence of factors other than gel filtration. Analogous to what was found for Phenyl-Corasil and for Poragel PN, it was found for Hydrogel IV that the peptides most retained are the larger peptides containing the most basic functions. This might indicate the presence on the polymer of acidic groups, giving rise to polar interactions.

DISCUSSION

The emphasis has been on the evaluation of packing materials for use in HPLC of peptides rather than on the evaluation of peptides for purity and nature of contaminants. Accordingly, few large-scale separations have been performed followed by analysis of the components of a sample. The power of HPLC is seen by the fact that the applied peptides, although proven to be TLC homogenous and to give correct amino acid analysis before HPLC, could be resolved into more than one component by suitable choice of HPLC conditions. In most cases, however, the peak attributed to the target peptide was by far the predominant peak in the 220-nm chromatogram; detection at 254 nm was ambiguous. The increased sensitivity for peptides at 220 nm makes this wavelength generally much more useful for detection of peptides than 254-nm detection and detection by refractive index.

Of the four HPLC packings used here, Phenyl-Corasil, Poragel PN, and Poragel PS are reversed-phase packing materials, and Hydrogel IV is a gel filtration packing. It was hoped that Hydrogel IV could provide a calibration curve correlating retention with molecular weight of the peptides. This calibration curve could then be used for an estimate of the molecular weight of physiologically active unknown peptides (for example, of hypothalamic origin) based on the retention of the physiological activity as determined by bioassay on different fractions from the column. For this purpose, the absence of factors other than gel filtration is preferred, but apparently this is not the case for Hydrogel IV, as evidenced by the very broad peaks and the excessive retention found for peptides containing basic amino acids. It is believed that both reversed-phase behaviour and especially polar interactions between acidic groups on the Hydrogel IV and basic groups on the peptide contribute significantly to retention. These effects seem to make Hydrogel IV unsuitable as a general gel filtration packing material for the determination of the molecular weights of peptides under the conditions used.

For the three reversed-phase packings, it was found that the retention of peptides was also affected by polar interactions. For Phenyl-Corasil the cause is assumed to be residual silanol groups in the silica support for the bonded organic phase. These silanol groups interact with basic groups on the peptides, and a similar type of interaction apparently also takes place on Poragel PN, presumably due to free carboxy groups on this gel. Phenyl-Corasil and Poragel PN were found to behave very similarly with respect to selectivity and retention of peptides, as well as to column efficiency, even though Phenyl-Corasil is a superficially porous packing material that would be expected to show a higher efficiency than the totally porous Poragel PN. Under the conditions used, these two packing materials are most suitable for peptides which do not have too much basic character, as this could lead to excessive retention, such as was experienced with the luteinizing hormone releasing hormone.

Poragel PS contains a basic functionality and is most suitable for peptides containing basic amino acids, as small acidic peptides without basic groups are too retained under the conditions used. The fact that Poragel PS, which like Phenyl-Corasil and Poragel PN is a reversed-phase packing material, shows an entirely different retention pattern, indicates that the main factor contributing to the retention of peptides on these columns is polar interactions between acidic and basic groups on the peptide and the packing material, rather than reversed-phase adsorption or partition.

None of the columns showed a very good efficiency although the plate number for Phenyl-Corasil and Poragel PN is about four times as large as the plate number for a similar Poragel PS column. Peaks from Phenyl-Corasil showed pronounced tailing, and Poragel PN showed weakly fronting peaks. This asymmetry in peak shape and the related dependency of retention upon sample load can be regarded as overloading phenomena, although such effects are not normally seen at the quite small sample loads used. A superficially porous packing is normally not expected[21] to overload at sample sizes less than *ca.* 0.1 mg sample per g packing material (equivalent to *ca.* 0.5 mg sample on a 3 ft. × 1/8 in. column), and the totally porous packing materials have an even larger capacity. The fact that *ca.* 50 μg peptide on a 3 ft. × 1/8 in. column caused overloading shows that the conditions used for HPLC of peptides were far from optimal. The usual way of improving this situation is by changing either the mobile phase or the packing material. Volatile buffers could be added to the aqueous eluants in order to minimize polar interactions. This could also be achieved with Phenyl-Corasil upon elimination of the residual silanol groups by silylation of the packing material with dimethyldichlorosilane or trimethylchlorosilane and hexamethyldisilazane as described[22]. Similarly, a reversed-phase packing material based on a support like Zipax (DuPont), which shows very weak adsorptive properties[21], could be used. Another approach would be to utilize a bonded phase packing with a more polar organic phase like alkyl ether (DuPont), alkylnitrile (DuPont, Varian, Waters) or carbohydrate (Corning, Waters), or ion exchange on one of the several ion exchangers specifically designed for HPLC.

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