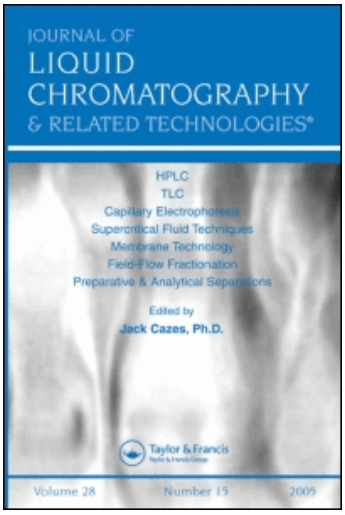

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FACTORS AFFECTING THE SEPARATION OF ARGININE VASOPRESSIN PEPTIDE DIASTEREOISOMERS BY HPLC

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ABSTRACT

Experimental conditions and parameters involved in HPLC separations of the peptide hormone arginine vasopressin and some of its diastereoisomers on several reverse phase columns were investigated. The effects of percent carbon loading on an octadecyl reverse phase column, carbon chain length of the bonded phase, concentration of the buffer, and organic solvent were examined. Using the appropriate solvent systems, arginine vasopressin was separated from each of its diastereoisomers with most solvent systems studied, but the order of elution of the diastereoisomers was dependent on the column employed. Separation of the peptides was only part of the goal. A continuing study to understand the interactions of the peptides with the stationary phase as a function of structure was also undertaken. This lead to the conclusion that the choice of column as well as solvent affect the separation of peptide diastereoisomers and that both the eluting strength of the mobile phase and the stationary phase chemical composition must be considered.

INTRODUCTION

There has been an increasing interest in the separation of moderate size peptides (700-3000 MW) by high performance liquid chromatography (HPLC) on both the analytical and preparative

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scale. To date, HPLC has been used for trace determination of specific peptides in physiological fluids and the purification of synthetic peptides for biological, chemical, and biophysical studies (1-4). In addition, recent studies in our laboratory indicate that the highly selective interactions of the peptides with the chromatographic stationary phase can be used to obtain information about the topological structure of these molecules (5). The synthesis of selected diastereoisomer analogs of the naturally occurring peptides has proven to be a valuable tool in evaluating the role played by certain amino acids in determining the biological activity of a variety of hormones (see for example, 4-9). Chromatographic studies of these diastereoisomers have been shown to provide unique insight into the changes in structure which occur upon substitution of a single D isomer into the peptide (5,8).

In this paper we present a chromatographic study using arginine vasopressin (Figure 1) (AVP) and several analogs where one amino acid has been changed to the D-configuration. AVP has a molecular weight of 1084 and is one of the neurohypophyseal hormones found in mammals including man. In position 1 there is a half-cystine, 2 tyrosine, 3 phenylalanine, 4 glutamine, 5 asparagine, 6 half-cystine, 7 proline, 8 arginine and position 9 glycinamide. The peptides studied include the naturally occurring all L configuration hormone and the following peptides with single amino acid substitutions with the D-configuration: [1-hemi-D-cystine]-AVP (1-Hemi-D-Cys), [2-D-tyrosine]-AVP (2-D-Tyr), [4-D-glutamine]-AVP (4-D-Gln), and [8-D-arginine]-AVP (8-D-Arg).

The experimental parameters and conditions for the reverse phase HPLC separation of the peptide diastereoisomers examined are presented. We have also examined the effects of reverse phase supports and solvents on the separation of the diastereoisomers. The data obtained provides further insight into the underlying physical-chemical processes which affect the separation of the

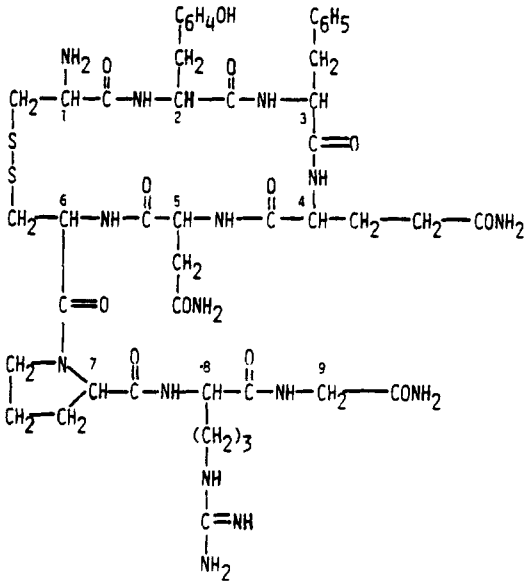

FIGURE 1. Structure of arginine vasopressin (AVP). The numbers (1,2,3, etc.) correspond to the residue from the N-terminal half cystine-1 to the C-terminal glycinamide-9.

diastereoisomers, and permits us to discuss these in terms of the differential topological structure of the hormones.

MATERIAL AND METHODS

Synthesis and Purification of Peptides

Arginine vasopressin (AVP) and most of the diastereoisomers of AVP used in this study were synthesized in our laboratory by the solid phase synthesis method (10). The solid phase methods were similar to those previously used in our laboratory for preparing arginine vasopressin and related analogs (11,12). The compounds were separated from related diastereoisomers and purified by partition chromatography on Sephadex G-25 (11,13) using purified solvents as previously reported (14). A final

purification on Sephadex G-25 using 0.2 N aqueous acetic acid as eluent solvent was always utilized. The sources for the various compounds are as follows: (1) arginine vasopressin was prepared and purified as previously reported (11); (2) [2-D-tyrosine, 8-arginine]-vasopressin (9,11); (3) [1-hemi-D-[α-$^2H_1$]cystine, 8-arginine]-vasopressin (11); (4) [8-D-arginine]-vasopressin was a generous gift from Dr. Maurice Manning, Department of Biochemistry, Medical College of Ohio at Toledo; (5) [4-D-glutamine, 8-arginine]-vasopressin was prepared by Dr. V. Viswanatha using the solid phase method, and N-Boc-D-glutamine with dicyclohexylcarbodiimide and N-hydroxybenzotriazole for coupling this protected amino acid to the growing peptide chain (15). The general methodology used for the rest of the synthesis followed closely the procedures of Yamamoto et al. (11). Following preparation of H-Cys(DMB)-Tyr(Bzl)-Phe-D-Gln-Asn-Cys(DMB)-Pro-Arg(Tos)-Gly-O-Resin, the peptide was cleaved from the resin by ammonolysis in anhydrous methanol. The protecting groups were removed by sodium in liquid ammonia, the peptide oxidized by 0.01 N $K_3Fe(CN)_6$, and then purified by partition chromatography on Sephadex G-25 in the usual manner (11) followed by gel filtration on Sephadex G-25. The purity of the peptides was established by quantitative amino acid analysis, thin layer chromatography in three or four solvent systems, optical rotation, elemental analysis, and by analytical HPLC analysis. The solvent systems used for (TLC) were 1-butanol-acetic acid-water (4:1:5, upper phase only); 1-butanol-acetic acid-pyridine-water (15:3:10:12); 1-pentanol-pyridine-water (7:7:6); and ethyl acetate-pyridine-acetic acid-water (5:5:1:3).

Reagents

The mobile phase used for all HPLC procedures was composed of a triethylamine-acetic acid buffer (TEA-HOAc) (Solvent A) combined with a water miscible organic solvent (Solvent B). Solvent A was prepared by diluting the desired volume of acetic

acid (J. T. Baker, Phillipsburg, NJ) in 950 mL of water and titrating the solution to a pH of 4.0 using triethylamine (TEA) (Mallinkrodt, St. Louis, MO) and then adjusting the total volume to 1 liter. It should be recognized that the pH values reported in this work are those of the aqueous buffer measured before addition of the organic modifier. The organic portion was either methanol (CH_3OH), acetonitrile (CH_3CN) or tetrahydrofuran (THF) (Burdick and Jackson, Muskegon, MI), used without further purification. The solvents were filtered and degassed as previously reported (5).

Apparatus

The HPLC equipment used was the same as that previously reported (5) except for the following. The peaks were monitored at 280 nm using the Model 440 absorbance detector and also were monitored at 215 nm using a Model 450 variable wavelength absorbance detector (Waters Associates, Milford, MA). The columns used in this study are described in Table 1.

TABLE 1
Column Characteristics

Column	% Carbon (by wt)	Endcapped (Y/N)	Column Dimensions	Flow rate (mL/min)
Spherisorb ODS(a)	5	N	250 x 3 mm id	2.0
μ-Bondapak C_{18}(b)	10	Y	300 x 3.9	2.0
LiChrosorb RP-18(c)	20	N	250 x 4.6	2.0
LiChrosorb RP-8(c)	12	N	250 x 2.1	1.0

(a) Phase Separation Ltd., Queensferry, Clwyd, UK.
(b) Waters Associates, Milford, MA.
(c) E. M. Laboratories, Darmstadt, Germany.

Chromatographic Study

The data was computed and reported as in our previous work (3). Retention measurements will be discussed in terms of the capacity factor, k', and in terms of the relative retention, α, which is the ratio of the k' of the particular diastereoisomer to that of the naturally occurring AVP.

The objective of this study was to see what effect changes in organic modifier, buffer strength and stationary phase composition would have on the relative retention of the diastereoisomers examined. The study consisted of the following experiments. First using a μ-Bondapak C_{18} column and holding the k' of AVP within a range of 5 to 7, the peptides were chromatographed using the solvent systems listed in Table 2. The second series of experiments examined the effect on α values when the percent carbon load of the stationary phase was varied. Holding the solvent system constant, (0.05 M TEA-HOAc combined with 10% THF), the diastereoisomers were also chromatographed on a LiChrosorb RP-18 and a Spherisorb ODS column (Table 1). Finally, we wished to determine what effect the hydrocarbon chain length on the stationary phase would have on the α values. The diastereoisomers were therefore chromatographed on a LiChrosorb RP-8 column using a mobile phase consisting of 0.05 M TEA-HOAc combined with 7% THF.

RESULTS

The series of experiments performed were designed to provide separation of the peptide diastereoisomers but more importantly to allow us to differentiate between the contribution of the mobile phase composition vs. that of the stationary phase composition to the elution order and selectivity. The variations in mobile phase composition which were studied are shown in Table 2. We observed that in most cases separation was obtained, but the selectivity of the system does change as a function of the organic modifier.

TABLE 2
Solvent Conditions on the μ-Bondapak C_{18} Column

	% Organic Phase	Conc. of Buffer (M)
CH_3OH	25	0.025
	25	0.050
CH_3CN	12	0.025
	12	0.050
THF	10	0.050
	7	0.025
	6	0.050
	6	0.075
	4(a)	0.075

(a) Injected only AVP, [8-D-Arg]-AVP, and [4-D-Gln]-AVP.

In order to allow for comparison from solvent to solvent, the capacity factor, k', for AVP was held relatively constant for the different mobile phases investigated. For purposes of identification each compound was injected both in a mixture and, if necessary, individually in order to confirm the order of elution.

The capacity factors and relative retentions for the peptides with methanol as the mobile phase organic modifier using the μ-Bondapak C_{18} column are shown in Table 3. The

TABLE 3
μ-Bondapak C_{18} Elution Profile with Methanol as the Organic Modifier

	25% CH_3OH 0.025 M TEA-HOAc pH 4.0		25% CH_3OH 0.05 M TEA-HOAc pH 4.0	
AVP Analog	k'	α	k'	α
AVP	5.99	1.00	5.17	1.00
8-D-Arg	7.07	1.18	6.07	1.17
4-D-Gln	7.26	1.21	6.43	1.24
2-D-Tyr	12.23	2.04	10.36	2.00
1-Hemi-D-Cys	27.84	4.65	25.27	4.89

value of 25% CH_3OH was used because it seemed to provide reasonable separation of the peptides with respect to time. The order of elution was found to be AVP > 8-D-Arg > 4-D-Gln > 2-D-Tyr 1-Hemi-D-Cys. Increasing the acetate concentration leads to a decrease in the retention volume of all species. Even though the capacity factors are decreased, the relative retentions remain essentially unchanged.

Table 4 shows the capacity factors and relative retentions of the peptides with acetonitrile as the mobile phase organic modifier. The value of 12% CH_3CN was used because it provided approximately the same capacity factor for AVP as did 25% CH_3OH. The order of elution remained unchanged. Again, an increase in the acetate concentration caused the peaks to be eluted faster, even though the relative retentions did not change significantly.

Table 5 shows the capacity factors and the relative retentions of the peptide with tetrahydrofuran as the mobile phase organic modifier. The percentage organic modifier was determined for reasons stated earlier. Again, the order of elution remained the same. The effects of decreasing the concentration of the organic modifier and increasing the buffer concentration is shown (Table 5) when changing from 7% THF, 0.025 M TEA-HOAc to 6% THF, 0.05 M TEA-HOAc. All of the peptides except 1-Hemi-D-Cys-AVP

TABLE 4
μ-Bondapak C_{18} Elution Profile with Acetonitrile as the Organic Modifier

	12% CH_3CN 0.025 M TEA-HOAc pH 4.0		12% CH_3CN 0.05 M TEA-HOAc pH 4.0	
AVP Analog	k'	α	k'	α
AVP	9.16	1.00	7.01	1.00
8-D-Arg	10.57	1.15	8.03	1.15
4-D-Gln	10.57	1.15	8.13	1.16
2-D-Tyr	16.29	1.78	12.36	1.76
1-Hemi-D-Cys	42.50	4.64	32.11	4.58

TABLE 5
μ-Bondapak C_{18} Elution Profile with Tetrahydrofuran as the Organic Modifier

	7% THF 0.025 M TEA-HOAc pH 4.0		6% THF 0.05 M TEA-HOAc pH 4.0		6% THF 0.075 M TEA-HOAc pH 4.0	
AVP Analog	k'	α	k'	α	k'	α
AVP	7.43	1.00	7.11	1.00	6.73	1.00
8-D-Arg	8.29	1.12	7.93	1.12	7.54	1.12
4-D-Gln	9.03	1.22	8.87	1.25	8.44	1.25
2-D-Tyr	16.56	2.23	15.84	2.23	14.87	2.21
1-Hemi-D-Cys	22.29	3.00	23.13	3.25	22.17	3.29

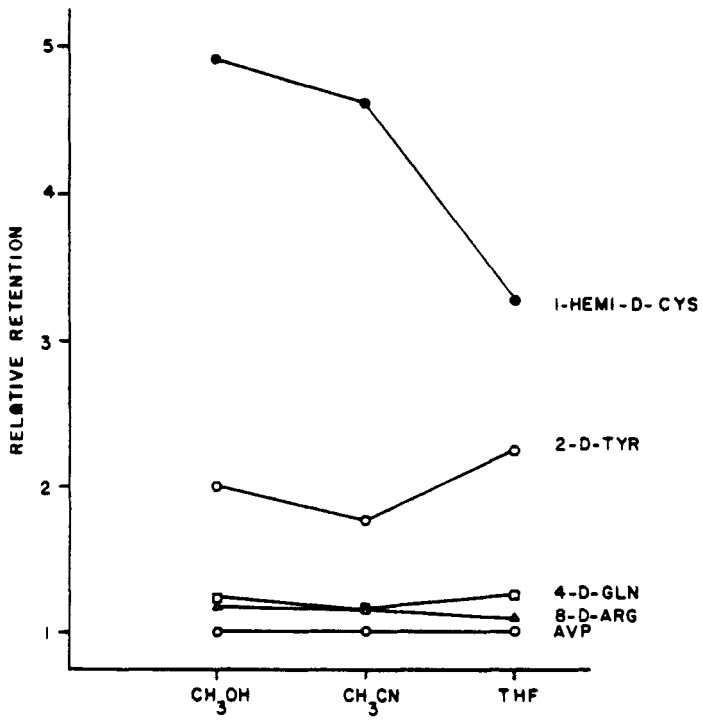

FIGURE 2. Plot of relative retention (α) vs. organic modifiers (25% CH_3OH, 12% CH_3CN and 6% THF) on the μ-Bondapak C_{18} column.

showed a slight decrease in k' and their retention relative to AVP remained constant. However, the 1-Hemi-D-Cys-AVP showed both an increase in its k' as well as in its relative retention. On the other hand, by keeping the percentage organic modifier constant (6%) and increasing the buffer concentration, the capacity factors decrease, but the relative retentions remain essentially constant.

Figure 2 shows the effect of the different organic modifiers with the same buffer concentration. The relative retentions of the peptides decrease when comparing CH_3OH to CH_3CN, but increase

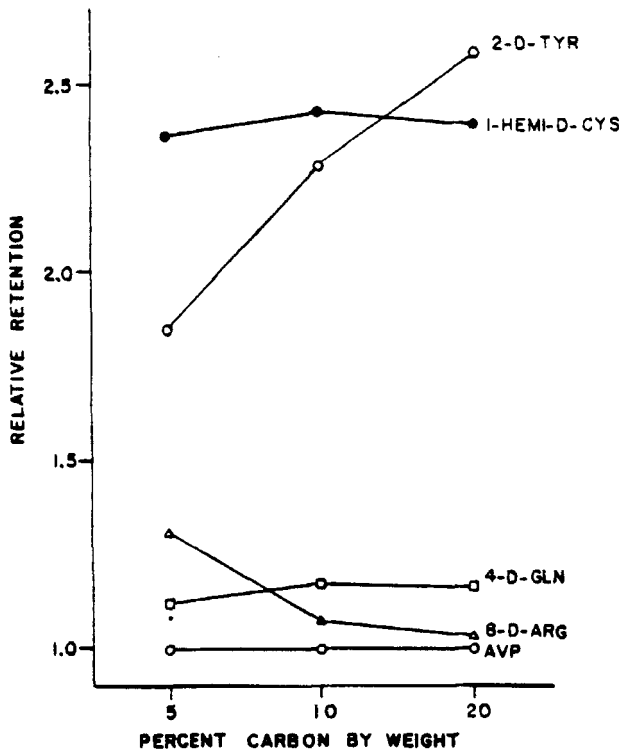

FIGURE 3. Plot of relative retention (α) vs. different C_{18} columns studied (Table 1) with 10% THF as the mobile phase organic modifier (Table 2).

for some when comparing to THF. Clearly solvent strength arguments are not adequate to describe this chromatographic behavior.

In the next series of experiments the diastereoisomers were subjected to chromatography holding the mobile phase constant and varying the hydrocarbon composition of the stationary phase. The columns investigated are shown in Table 1. The elution order of the diastereoisomers was shown to be dependent on the amount of C_{18} hydrocarbon bonded to the surface of the support (Figure 3). The elution order on the μ-Bondapak C_{18} (Table 1) column is (Figure 3) AVP > 8-D-Arg > 4-D-Gln > 2-D-Tyr > 1-Hemi-D-Cys. On the Spherisorb ODS column the 4-D-Gln- and 8-D-Arg-AVP reverse elution order and on the LiChrosorb RP-18 column the 2-D-Tyr- and 1-Hemi-D-Cys-AVP reverse elution order.

The elution profile of the LiChrosorb RP-8 column (Table 1) with 7% THF and 0.05 M TEA-HOAc pH 4.0 compared to the μ-Bondapak C_{18} column with 7% THF and 0.025 M TEA-HOAc pH 4.0 is shown in Figure 4. It was observed that the elution order of 4-D-Gln- and 8-D-Arg-AVP was reversed. If one compares the elution order and the relative retention of the C_8 column (Figure 4) to the Spherisorb ODS column (Figure 3), no significant changes are observed.

Figure 5 shows a typical chromatogram of the separation of the peptides on the μ-Bondapak C_{18} column using 6% THF and 0.075 M TEA-HOAc pH 4.0 as the mobile phase. To further show the separation of AVP, 8-D-Arg-, and 4-D-Gln-AVP, the percentage THF was decreased to 4% (Table 2) and the chromatogram in Figure 6 was obtained. Even though the retention time was increased, the relative retention remained unchanged.

Adjusting the pH to 6.0 caused a drastic increase in retention for all species and a loss of all resolution (unpublished results).

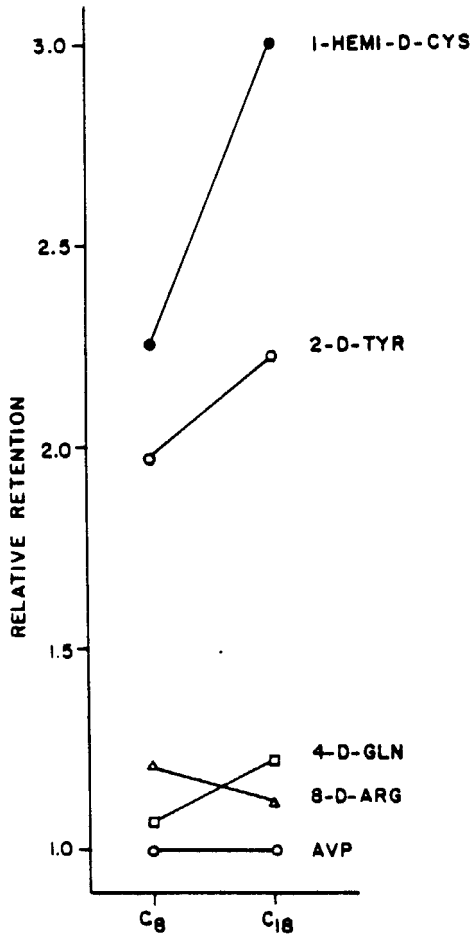

FIGURE 4. Plot of relative retention (α) vs. different hydrocarbon chain lengths comparing the LiChrosorb RP-8 and the μ-Bondapak C_{18} columns (Table 1) with 7% THF as the mobile phase organic modifier.

DISCUSSION

Chromatographic systems which utilize a hydrocarbon bonded to a solid silica support material as an adsorbent are commonly referred to as "reverse phase" systems. Reverse phase simply

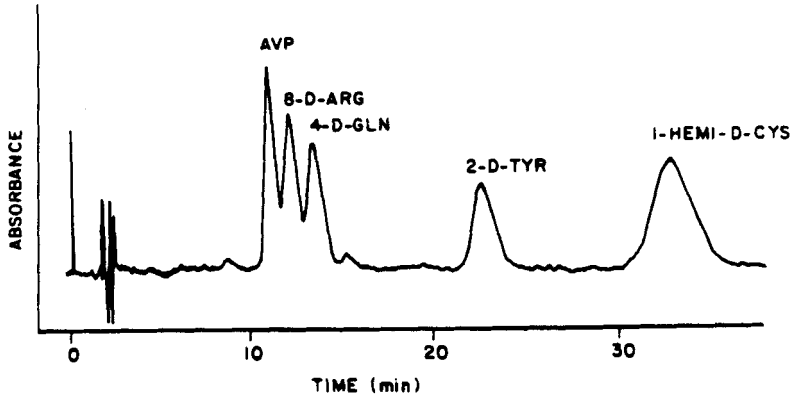

FIGURE 5. Chromatogram of the AVP analogs studied on the μ-Bondapak C_{18} column with 6% THF as the organic modifier and 0.075 M TEA-HOAc buffer as the aqueous portion of the mobile phase.

implies that the effective stationary phase is less polar than the mobile phase. In order to discuss the interactions between the solute molecules and the mobile and stationary phases, which are responsible for the chromatographic selectivity, it is necessary to recognize the chemical and physical nature of the

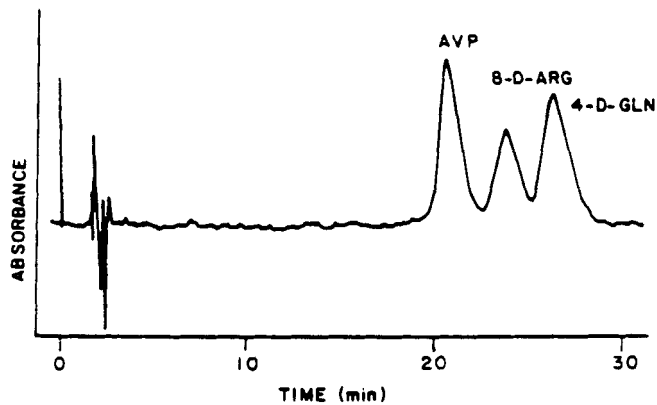

FIGURE 6. Chromatogram of AVP, 8-D-Arg-AVP and 4-D-Gln-AVP on the μ-Bondapak C_{18} column with 4% THF and 0.075 M TEA-HOAc buffer mobile phase.

effective stationary phase. The stationary phase consists of the solid support and the bonded hydrocarbon both of which are solvated by the components of the mobile phase as depicted in Figure 7. Therefore, a change in the chemical composition of the mobile phase not only changes the eluting strength of this phase but also changes the retentive nature of the stationary phase. The chromatographic behavior of a compound in different solvent systems must, therefore, be considered in terms of changes occurring in both phases (16,17).

The effects of changing the buffer concentrations with different organic modifiers were investigated. The μ-Bondapak C_{18} column (Table 1) was used for this study. In all cases it was observed that an increase in buffer concentration results in a decrease in the capacity factors. The more rapid elution can be explained by a greater solubility of the peptides in the mobile phase at the higher concentration of buffer. Even though k's change, the relative retentions do not change significantly. This indicates therefore that the selectivity is determined by the stationary phase.

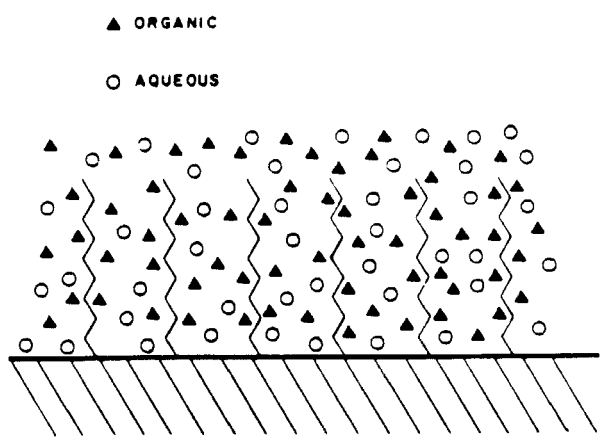

FIGURE 7. A pictorial representation of the solvated bonded stationary phase.

A comparison of the retention parameters of these species as a function of the different organic modifiers with the same aqueous buffer (Figure 2) shows that the absolute retention of the AVP and its diastereoisomers is a function of the organic modifier solvent strength (18). The capacity factor for AVP is held to a value of 5 to 7 by adjusting the fraction of organic modifier used. However, the relative retention of the diastereoisomers is seen to vary depending on the choice of organic modifier. For example, even though THF is considered a stronger solvent than either CH_3OH or CH_3CN, 2-D-Tyr-AVP has an increase in its relative retention in THF. A more precise way to interpret this behavior is to consider that THF and CH_3CN are more effective in solvating the bonded phase than is CH_3OH. This solvation of the surface determines the chemical composition of the effective stationary phase. Interactions of the solute molecules with the stationary phases therefore will be a function of the amount of hydrocarbon bonded to the surface and the components of the mobile phase associated with both the silica substrate and the bonded hydrocarbon.

The next parameter investigated was the effects of different percent carbon of the bonded phase (Table 1). Previous work in our laboratory has shown that there is a significant difference in the effective concentrations of hydrocarbon in the stationary phase of the three C_{18} packing materials (16,17). The data shown in Figure 3 demonstrates that the order of elution as well as the retention of the diastereoisomers relative to the parent compound is a function of the amount of hydrocarbon bonded to the surface. The lightly loaded Spherisorb ODS (5% C) provided the greatest dispersion, i.e. difference in relative retention of all of the diastereoisomers. The μ-Bondapak C_{18} column (10% C) showed an increase in the retention of both the 2-D-Tyr- and the 1-Hemi-D-Cys-AVP and a decrease in the retention of the 8-D-Arg-AVP. The increase in attraction of the 2-D-Tyr- and 1-Hemi-D-Cys-AVP is a very strong indication that the conformations of

these diastereoisomers provide a greater exposure of lipophilic moieties. This increased exposure interacts more strongly with the stationary phase which has been made more non-polar by the increase in the hydrocarbon content. The decrease in the relative retention of the 8-D-Arg-AVP implies that this diastereoisomer becomes more like the native hormone in its ability to interact with a non-polar environment.

Further increasing the hydrocarbon content of the stationary phase (see the LiChrosorb RP-18 column (20% C)), which also increases the amount of organic modifier associated with the bonded phase, provides an even more non-polar stationary phase. These changes result in the 8-D-Arg-AVP eluting even closer to the native hormone indicating that the lipophilic properties of the two molecules are very similar. The increase in the relative retention of the 2-D-Tyr analog can be explained on the basis that the aromatic groups are more exposed and thus are more available for interaction with the lipophilic stationary phase. This increase in the lipophilic nature is consistent with the Nuclear Magnetic Resonance (NMR) studies of the conformational properties of these molecules (19,20). For example, ^{1}H NMR studies indicate the aromatic rings of Tyr-2 and Phe-3 in AVP interact intramolecularly (19), while the ^{13}C NMR studies (20) of 2-D-Tyr-AVP indicate the interaction is disrupted, making the lipophilic side chains at positions 2 and 3 more accessible for interaction with the stationary phase in this diastereoisomer. The much longer retention time for 1-Hemi-D-Cys-AVP relative to AVP indicates that by changing the relationship of the Half-Cys-1 terminal amino groups (which is charged at the pH of this study) and the Tyr-2 side chain, the latter lipophilic side chain becomes much more accessible for interaction with the lipophilic stationary phase. This is consistent with circular dichroism studies which have suggested that local structural rigidity in this part of the AVP molecule is in part a result of interaction of the 1 and 2 residues (21,22), and ^{13}C NMR

studies (20) which indicates that this structural feature is disrupted in the 1-Hemi-D-Cys analog. The fact that the 1-Hemi D-Cys analog is now retained less than the 2-D-Tyr analog, shows how sensitive the interaction of these molecules with a non-polar phase is to changes in this position. However, it is interesting to note that the retention time of the 1-Hemi-D-Cys analog is not affected by changes in the hydrocarbon content of the stationary phase. This is undoubtedly due to a trade off between the greater attractive interaction between the more exposed Tyr-2 side chain and the repulsive interaction of the charged, and more exposed, terminal amino group in the 1-Hemi-D-Cys analog, which results in an increased but relatively constant interaction of the molecule with the different stationary phases.

The 4-D-Gln diastereoisomer is only slightly affected by the increase in the non-polar nature of the stationary phase. While it is more strongly retained than the native hormone, its chromatographic behavior does not represent a large increase in selective interaction. This suggests that the conformation of this diastereoisomers and of AVP are very similar, as was also reported for the 4-D-Gln-oxytocin and oxytocin case (5).

The next variable examined was the effect of the hydrocarbon chain length. This was done by comparing the elution of the diastereoisomers from the μ-Bondapak C_{18} column to that of the LiChrosorb RP-8 column (Table 1). As seen in Figure 4 the relative retentions of the peptides are less on the RP-8 column except for 8-D-Arg-AVP. The 8-D-Arg-AVP is more strongly attracted to the C_8 stationary phase than to the μ-Bondapak C_{18} stationary phase. If one compares the ODS column (Table 1) to RP-8 column, it is observed that the order of elution does not change and the relative retentions remain essentially constant. This would suggest that the 12% carbon-by-weight in the C_8 column is similar to the 5% carbon-by-weight ODS column. While the C_8 column has approximately twice as much hydrocarbon bonded to the surface as does the ODS column (5% C) the composition of the

stationary phases on these two columns is quite similar. The very different retention of these species on the μ-Bondapak C_{18} column (10% C) clearly shows the carbon chain length of the bonded phase along with its surface coverage determines the composition of the stationary phase but that the chain length itself does not determine the specific interaction.

The type of information obtained chromatographically is complementary to other techniques used for studying topochemistry, and gives a good indication of the chemical interactions possible. Further studies on a heavily loaded bonded phase which can represent a lipid-like layer needs to be performed. If one is to use the chromatographic behavior of the molecule as a diagnostic tool, it will be necessary to have a more complete understanding of the chemical nature of the stationary phase.

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