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SEPARATION OF AMINO ACIDS AND PEPTIDES ON NON-POLAR STATIONARY PHASES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Microparticulate non-polar stationary phases, such as octadecyl-silica offer a rapid and efficient means for the separation of peptides and amino acids by high-performance liquid chromatography. Retention is attributed to hydrophobic interaction between the solutes and the hydrocarbonaceous functions covalently bound to the stationary phase surface. Consequently the species are eluted in the order of increasing hydrophobicity. Various peptide mixtures were analyzed by using gradient elution with increasing acetonitrile concentration in the eluent and monitoring the column effluent at 200 or 210 nm with an UV detector. The separation of angiotensins and enzymic digest of polypeptides illustrates the speed of the method which can be used to assay the purity of peptide hormones such as α-melanotropin and gramicidin or to analyze the composition of reaction mixtures involving peptides. The efficiency of the method is superior to that obtained on the conventionally used ion-exchanger columns, except for hydrophilic amino acids and peptides that are poorly retarded. Nevertheless, with a suitable ionic surfactant in the mobile phase, non-polar stationary phases can be used for the separation of these species as well.

INTRODUCTION

The adsorption of amino acids and peptides on charcoal was first investigated by Abderhalden and Fodor[1], and this non-polar sorbent was widely used in the chromatographic separation of such species in the forties[2–4]. The subsequent development of ion-exchange chromatography[5,6], however, largely reduced the significance of all other liquid–solid chromatographic techniques in this particular field. Commercially available amino acid analyzers equipped with ion-exchange resin columns and a ninhydrin reactor have been widely used not only for the analysis of amino acids, but also for that of small peptides[7]. Large peptides have usually been separated on weak ion exchangers, such as carboxymethylcellulose[8], by gel filtration[9], or by bioaffinity chromatography[10]. Although major efforts were made to advance gas chromatography for the analysis of amino acids and small peptides[11], ion-exchange chromatography has remained the preeminent chromatographic technique.

Recent progress in high-performance liquid chromatography (HPLC) has revived the interest in non-polar stationary phases. In fact, the presently most widely used chromatographic technique, which is often referred to as "reversed-phase" chromatography, employs silica gel with covalently bound hydrocarbonaceous functions, such as octadecyl moieties, as the stationary phase. In the course of our research to apply this type of chromatography to the separation of polar biological substances[12–15], we found that with recently available high-efficiency columns, the technique is eminently suitable for the rapid separation of complex peptide mixtures and shows promise as a tool for amino acid analysis.

In this report we wish to illustrate the speed and efficiency of "hydrophobic" chromatography in the separation of amino acids and peptides. The use of hydrocarbonaceous stationary phases is not only convenient and yields reproducible results, but under proper conditions may also facilitate the estimation of retention behavior from "hydrophobicity" of the solutes, which are available from the literature.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Model 601 high-pressure liquid chromatograph with a Schoeffel FS-770 variable-wavelength UV detector and a Schoeffel FS-970 fluorescence detector was used. The sample was introduced by using a Siemens high-pressure injection syringe with a 10-μl needle or with a Rheodyne Model 7105 sample injection valve.

Columns

Preliminary experiments were carried out with home-made and commercial 5- and 10-μm octadecyl-silica columns. Most of the results presented here were obtained with 5-μm LiChrosorb RP-18 columns, 25 cm $\times$ 4.6 mm I.D. $\times$ 6.4 mm O.D., No. 316 stainless steel. In some experiments 5-μm LiChrosorb RP-8 columns of the above dimensions, but with octyl-silica packing instead of octadecyl-silica, were also employed. Both LiChrosorb columns were supplied by Rainin Instruments (Boston, Mass., U.S.A.).

Operating conditions

The flow-rate and column temperature were in most experiments 2.0 ml/min and 70°, respectively. Isocratic elution was carried out with 0.1 *M* phosphate buffer, pH 2.1 or 0.6 *M* perchloric acid, pH 0.2, without organic solvent in the eluent. In the gradient elution mode the above phosphate buffer and acetonitrile were used as the starting eluent and the gradient former, respectively, with the precautionary measures described previously[15]. Reagent grade chemicals (Fisher, Pittsburgh, Pa., U.S.A.) and "distilled-in-glass" grade acetonitrile (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.) were used exclusively. Distilled water was prepared with a Barnstead distilling unit in our laboratory.

The shapes of the gradients employed in this study are shown in Fig. 1 by plots of the concentration of organic solvent in the eluent at the column inlet as a function of time for different eluent gradients from water with methanol as the gradient former. The data were obtained by replacing the column with a short capillary

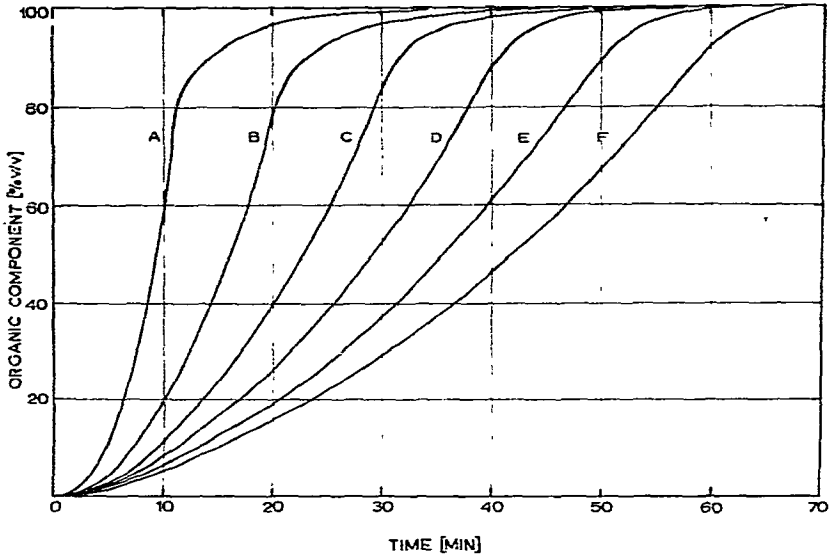

Fig. 1. Shape of the eluent gradients used in this study as shown by plots of methanol concentration in water. The curves were obtained at a concave curvature 0.3 with the following time settings: A, 10; B, 20; C, 30; D, 40; E, 50; and F, 60 min from 0 to 100% of the gradient former concentrations.

tube and monitoring the optical density of the liquid stream at 210 nm at various gradient settings on the instrument panel.

In some experiments the separation of hydrophilic amino acids was caried out at 25° with $3 \cdot 10^{-3}$ *M* decyl sulfate (Fisher) in the phosphate buffer employed in isocratic elution.

RESULTS AND DISCUSSION

In two recent papers[13,14] we have proposed a theoretical framework for the treatment of solute retention in liquid chromatography with non-polar stationary phases. It has been shown that, albeit the energetics of solute interaction with the hydrocarbonaceous functions of the stationary phase is complex, the balance of two antagonistic free energy changes is of particular significance in determining the value of the capacity factor in a given column. The first has been related to the decrease in the molecular surface area, which is exposed to the solvent, upon binding of the solute to the stationary phase and the effective surface tension of the eluent. The other has been expressed by the sum of the free energy changes arising from the interaction of the solute with the solvent molecules.

The free energy change associated with the reduction of the molecular surface area is proportional to the contact area between the solute and the hydrocarbonaceous moiety of the stationary phase and the effective surface tension. In most cases, for members of a homologous series, which contain the same polar functions, the energy of interaction with the polar eluent is about the same. Consequently, their retention order is essentially determined by the contact area, and therefore, for small molecules, it is proportional to the size of the molecules. This behavior manifests itself by the

theoretically predicted and experimentally observed linear relationship between the logarithm of the capacity factor, k', and the carbon number of the homologues.

Since water has the highest surface tension among all common solvents, maximum retention of a solute on a non-polar stationary phase can be obtained with neat aqueous eluents. On the other hand, the surface tension of hydro-organic mixtures decreases with increasing organic solvent concentration so that the capacity factor of a given eluite can be reduced by increasing the concentration of an organic solvent such as acetonitrile or methanol in the eluent.

Generally, the more "polar" a substance, the greater is its countervailing interaction energy with a polar solvent such as water. Consequently, the introduction of "polar" moieties into molecules results in a decrease in chromatographic retention under a given set of conditions. The ionization of certain groups in the solute molecule, which can be brought about by adjusting the pH of the eluent, has a similar effect. In contrast, retention can be enhanced by suppressing the ionization of the solute.

Chromatographic data obtained with biological substances over a wide range of chromatographic conditions have been quantitatively interpreted in the light of the theory[13,14]. Yet, the analysis has been restricted to isocratic elution conditions, and the elucidation of retention behavior in gradient elution, in which the solvent properties and consequently the free energy of solute–solvent interaction change in the course of elution, requires further study. However, for the separation of amino acids and peptides, having wide ranging "polarity", the gradient elution mode is necessary. Although the theory of gradient elution in liquid chromatography is well advanced[16], it requires the definition of the eluent strength, which will be the subject of a forthcoming investigation[17]. In the present work concave gradients have been used as shown in Fig. 1, because this gradient shape yielded optimum resolution in most instances. Furthermore, we found that the adjusted retention times for members of homologous series such as the oligomers of phenylalanine were linearly dependent on the number of residues. This observation suggests that with the gradients the "eluent strength" is an approximately linear function of time. The use of such gradients facilitates the interpretation of the relation between the chemical structure characterized by the hydrophobicity of the solute and its retention behavior under conditions of gradient elution.

Amino acids

In most cases, the separation of non-polar amino acids could be carried out with isocratic elution as shown in Fig. 2.

A homologous series of α-amino acids having n-alkyl side chains has been separated on octadecyl-silica with neat aqueous eluents of pH 2.1 and 0.2. As the pK_a value of the carboxylic groups in these species is between 2 and 3, the retention values are expected to be higher at the lower eluent pH because under such conditions the carboxylic groups are protonated. The results obtained by isocratic elution are shown in Fig. 3, which illustrates the linear relationship between the log k' values and the carbon number of the side chain. The ratio of the capacity factors of the undissociated species (at pH 0.2) to those of the about half dissociated species (at pH 2.1) is approximately two, in agreement with earlier observations that the capacity factor of protonated carboxylic acid decreases by a factor 3 to 4 upon complete dis-

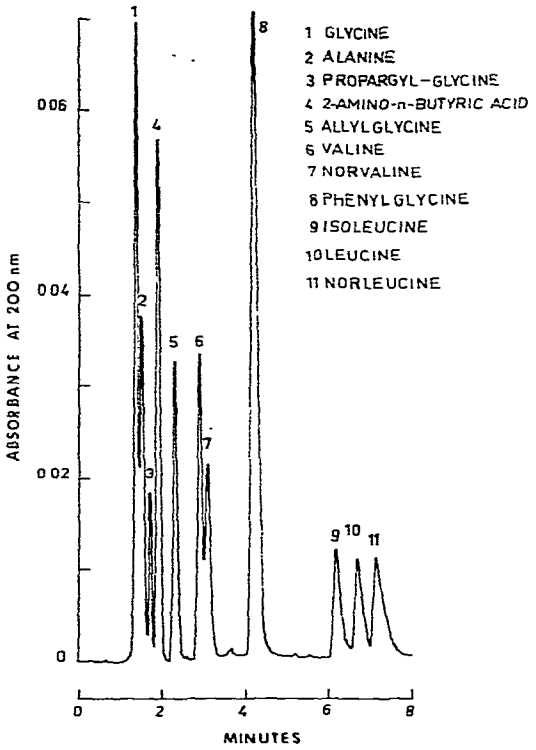

Fig. 2. Chromatogram of non-polar amino acids. Column: 5-μm LiChrosorb RP-18; 25 cm × 4.6 mm I.D.; eluent, 0.5 *M* $HClO_4$, pH 0.2; temperature, 70°; flow-rate, 2.0 ml/min; ΔP, 150 atm.

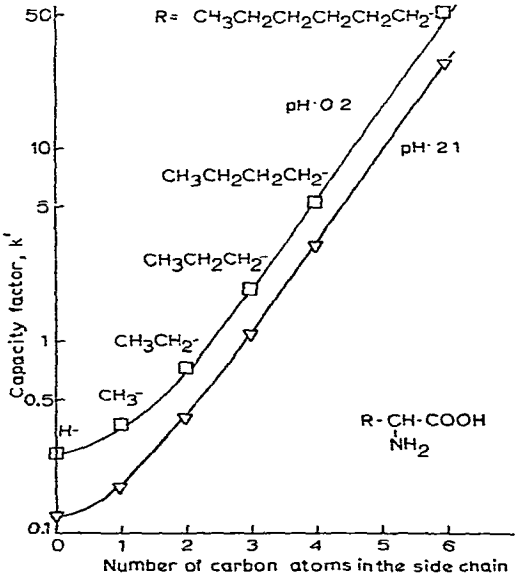

Fig. 3. Plots of the logarithm of the capacity factor against the side chain carbon number of *n*-alkyl-α-amino acids. Column and conditions are given in Fig. 2. The data at pH 2.1 were obtained with 0.1 *M* phosphate buffer.

sociation. The slopes of the straight-line portions of the plots are in qualitative agreement with the values predicted from the previous theoretical treatment[13,14].

The structure of the protein amino acids, which are of the greatest biochemical interest, is more complex, however, than that of the model compounds shown above. Their "polarity" can be widely different owing to the great diversity of the side chain functions. Since the importance of hydrophobic interactions in biochemistry has been recognized[18], these amino acids are frequently characterized as hydrophobic or hydrophilic. In order to express this property quantitatively, Nozaki and Tanford[19] used the free energy of transfer from water to ethanol or dioxane as a measure of hydrophobicity. Hansch and co-workers[20] have measured the partition coefficient, P, of amino acids in an octanol–water system and used log P to quantify their hydrophobic properties. Rekker[21] has presented a hydrophobicity scale for the side chains of various amino acids on the basis of hydrophobic fragmental constants. Although the three scales are not colinear, they show about the same order for the relative hydrophobicity of amino acids as shown in Table I.

TABLE I

HYDROPHOBICITY SCALES FOR PROTEIN AMINO ACIDS

P = Partition coefficient in octanol–water[19]; Δf_t = free energy of transfer of the side chain from pure organic solvent to water at 25°[20]; Σf = relative lipophilicities of the side chains according to Rekker[21].

Amino acid	*Symbol*	*log P*	Δf_t *(cal/mol)*	Σf
Tryptophan	Trp	−1.04	3400*	2.31
Phenylalanine	Phe	−1.43	2500*	2.24
Leucine	Leu	−1.71	1800*	1.99
Isoleucine	Ile	—	—	1.99
Tyrosine	Tyr	−2.26	2300*	1.70
Valine	Val	−2.10	1500**	1.46
Cystine	Cyst	—	—	1.11
Methionine	Met	—	1300**	1.08
Proline	Pro	—	—	1.01
Cysteine	Cys	—	—	0.93
Arginine	Arg	−2.59	—	—
Alanine	Ala	−2.94	500**	0.53
Lysine	Lys	−2.82	—	0.52
Glycine	Gly	−3.03	—	0.00
Aspartic acid	Asp	—	—	−0.02
Glutamine	Glu	—	—	−0.07
Histidine	His	−2.86	500*	−0.23
Threonine	Thr	—	400**	−0.26
Serine	Ser	—	−300**	−0.56
Asparagine	Asn	—	—	−1.05
Glutamine	Gln	—	—	−1.09

* Average of the values obtained with dioxane and ethanol.

** Values with pure ethanol.

The chromatograms in Fig. 4 illustrate the separation of protein amino acids on octyl- and octadecyl-silica columns. It is seen that hydrophilic amino acids are only slightly retarded on such non-polar stationary phases even with neat aqueous

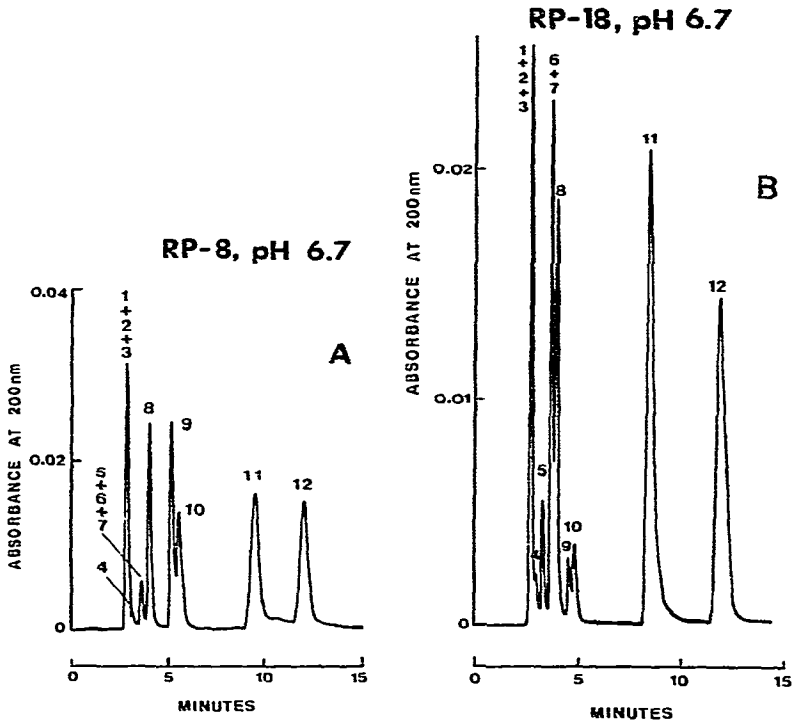

Fig. 4. Chromatograms of protein amino acids on (A) octyl- and (B) octadecyl-silica stationary phases. Columns: LiChrosorb RP-8 (5-μm octadecyl-silica) and LiChrosorb RP-18 (5-μm octadecyl-silica); eluent, 0.1 *M* phosphate buffer, pH 6.7; temperature, 70°; flow-rate, 2 ml/min; ΔP, 150 atm. Eluites: 1, glycine; 2, lysine; 3, proline; 4, alanine; 5, valine; 6, methionine; 7, cystine; 8, tyrosine; 9, isoleucine; 10, leucine; 11, phenylalanine; 12, tryptophan.

eluents. Consequently, the resolution of the polar amino acids is not sufficient by this technique for the analysis of protein hydrolyzates.

Both chromatographic retention on non-polar stationary phases with aqueous eluents and partitioning between organic solvents and aqueous solution have a similar physico-chemical basis[17]. It is expected then that the widely used log *P* values and the log *k'* values, obtained from chromatographic experiments, for various amino acids show a linear relationship. Indeed, a plot of the log *P* values obtained from the literature[20] against the logarithm of the capacity factors measured on octylsilica at pH 6.7 yields a straight line as shown in Fig. 5. This finding suggests that HPLC with non-polar stationary phases can be a convenient and effective tool to obtain data for use in quantitative structure–activity relationships[21].

Separation of small peptides

Isocratic elution. Small peptides consisting of hydrophilic amino acids such as glycine are only slightly retarded on the non-polar phases employed even with neat aqueous eluents. For instance, the oligomers of glycine containing up to six residues had no appreciable retention on octadecyl-silica when phosphate buffer, pH 2.1, was used as the eluent at 70°. This finding suggests that the peptide chain proper has no

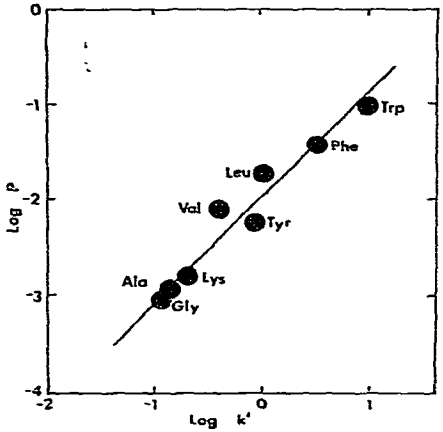

Fig. 5. Plot of the logarithm of the partition coefficient in octanol–water, P, against the logarithm of the capacity factor for some protein amino acids. The capacity factors have been measured on LiChrosorb RP-8 (5-μm octyl-silica) column under conditions stated in Fig. 4.

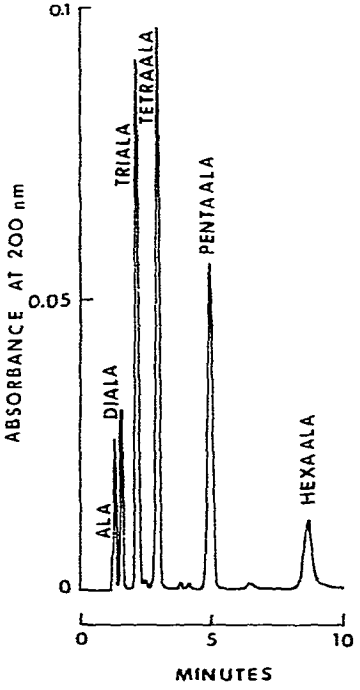

Fig. 6. Chromatogram of alanine oligomers. Column: 5-μm LiChrosorb RP-18; eluent, phosphate buffer, pH 2.1; temperature, 70°; flow-rate, 2 ml/min; ΔP, 150 atm.

or only a very small contribution to the retention of peptides under such conditions. The oligomers of L-alanine, however, can be well separated as shown by the chromatogram in Fig. 6. The plot of log k' against the number of alanine residues yields a straight line as illustrated in Fig. 7. Nevertheless, the slopes of the straight lines are much smaller than the slopes obtained with alkyl-α-amino acids under identical

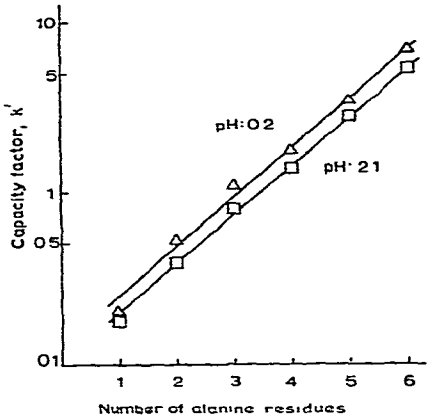

Fig. 7. Plots of the logarithm of the capacity factor against the number of residues in alanine oligomers. The chromatographic conditions were the same as in Fig. 3.

conditions and are shown in Fig. 3. The log k' increment of a methyl group of the aliphatic side chain is roughly twice as large as the log k' increment of a $-NH-CH(CH_3)CO-$ residue. Whereas our previous theoretical treatment[12,13] can account qualitatively for this difference, further studies are required to develop a quantitative framework for the interaction of peptide molecules with the hydrocarbonaceous ligands of the stationary phase under different elution conditions.

Gradient elution. In order to increase the peak capacity[22] in the separation of mixtures containing hydrophobic amino acids and peptides gradient elution at increasing concentration of acetonitrile was employed. The usefulness of this approach is exemplified by the chromatogram in Fig. 8. The change of "eluent strength" was such that the adjusted retention times of phenylalanine and its oligomers were a linear function of the number of residues. A good correlation was also found between the retention times of the various peptides and the sum of the side-chain "hydrophobicity numbers" given by Rekker[21]. This finding suggests that the retention order of peptides may be estimated from data pertinent to the amino acid constituents. Such an approach[23], which used the retention values of the individual amino acids to predict the retention of peptides in paper chromatography, was quite successful.

Retention behavior of peptides

Whereas the existing theories can be used to interpret qualitatively the retention of peptides, further detailed study is required to gain insight into the interaction between the hydrocarbonaceous stationary phase and solutes having such a complex architecture. Generally, the introduction of a polar amino acid residue reduces, whereas that of a hydrophobic amino acid residue increases retention, which also is influenced by the number and, in certain instances, the position of charged groups in the side chains. The situation appears to be similar to that observed with small molecules, *i.e.*, the introduction of a polar group, which interacts with the solvent, reduces retention, whereas the introduction of a non-polar group, which increases the contact area between the solute and the stationary phase, will increase retention. If ionogenic groups are present in the side chains, the ionization of these groups reduces

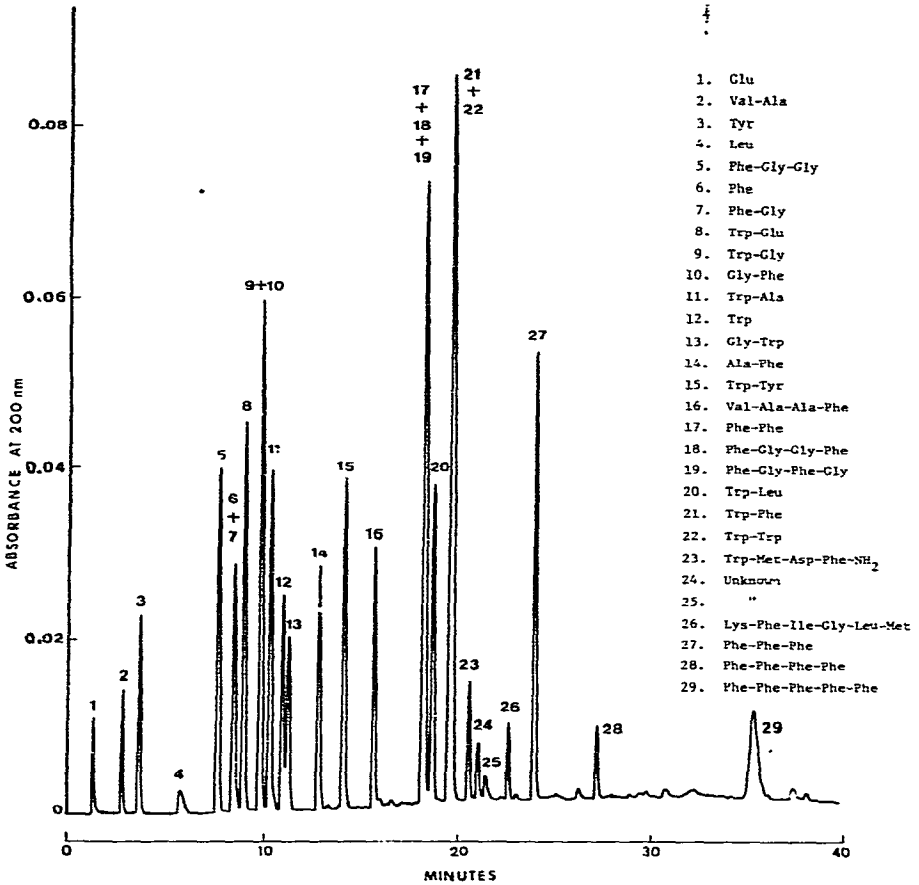

Fig. 8. Chromatogram of non-polar amino acids and small peptides. Column: 5-μm LiChrosorb RP-18; gradient elution from 0.5 *M* $HClO_4$, pH 0.2, with acetonitrile as the gradient former, Type E (*cf.* Fig. 1); temperature, 70°; flow-rate, 2 ml/min; initial ΔP, 150 atm. Sample: 10 μl containing *ca.* 1 μg of each component, see Table I for symbols.

retention. The use of hydro-organic solvents which have a lower effective surface tension than water also results in a reduction in chromatographic retention. These considerations are expected to apply to polypeptides which have a three dimensional structure since the fundamental features of the underlying theory have been shown to hold for proteins[24].

It is likely that the retention of a relatively large peptide could be estimated from the retention values of the amino acid residues obtained under identical conditions. This approach, however, has not been tested because, in our system, the capacity factor of the most hydrophilic amino acids was very small, and therefore their retention could not be quantified with sufficient accuracy. With increasing chain length the conformation of the peptide has also to be taken into consideration. HPLC on non-polar stationary phases appears to be a particularly attractive physico-chemical tool for gathering quantitative information on the structure and hydrophobic properties of such biological substances.

Chromatography of peptide hormones

The method has been employed to analyze commercial gramicidin claimed to have 100% biological activity. This peptide is a derivative of gramicidin A and has the following amino acid sequence: HCO-(L-Val)-(D-Gly)-(L-Ala)-(D-Leu)-(L-Ala)-(D-Val)-(L-Val)-(D-Val)-(L-Trp)-(D-Leu)-(L-Trp)-(D-Leu)-(L-Trp)-(D-Leu)-(L-Trp)-$CONHCH_2CH_2OH$. It is a highly hydrophobic peptide consisting of alternating L- and D-amino acids, which is assumed to have a helical structure with the hydrophobic side chains at the outside. The chromatogram of this substance is shown in Fig. 9. It has been established that the major peak and the circumadjacent four peaks are sample components whereas the other peaks are artifacts arising from trace impurities in the starting eluent. The preliminary results from an ongoing investigation using isocratic elution suggest that the five peaks represent various conformers of gramicidin and their relative concentrations are a function of pH and the concentration of organic solvent. It appears that HPLC with non-polar stationary phases can be a useful tool in the study of the thermodynamic and kinetic aspects of conformational changes which manifest themselves in the presence of conformers having different hydrophobicities.

Synthetic peptides of the angiotensin type, whose amino acid sequence is given in Table II, have been separated as shown in Fig. 10. The peaks representing the peptides have been identified by chromatographing each substance individually

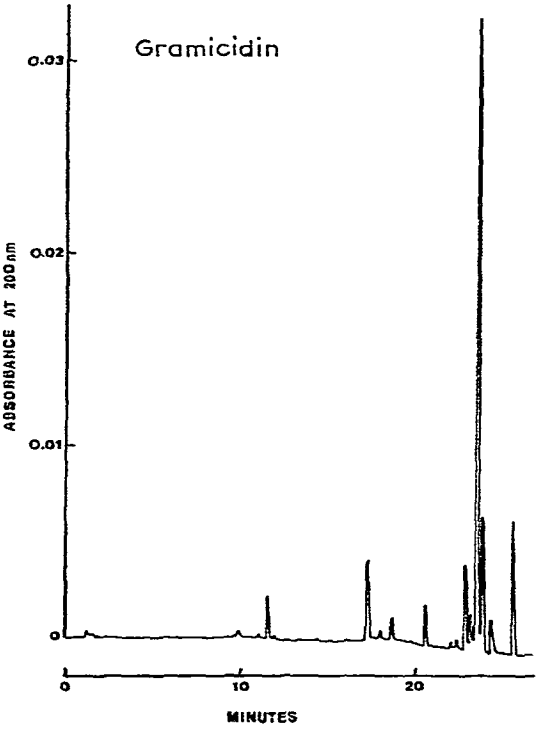

Fig. 9. Chromatogram of gramicidin. Column: LiChrosorb RP-8 (5-μm octadecyl-silica); gradient elution from 0.1 *M* phosphate buffer, pH 2.1, with acetonitrile as the gradient former; gradient shape, type D (*cf.* Fig. 1); temperature, 70°; flow-rate, 2 ml/min; initial ΔP, 150 atm.

TABLE II

AMINO ACID SEQUENCE FOR ANGIOTENSINS

No.	*Amino acid sequence*
1	Asn-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu
2	Asn-Arg-Val-Tyr-Val-His-Pro-Phe
3	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
4	Arg-Val-Tyr-Ile-His-Pro-Phe
5	Val-Tyr-Ile-His-Pro-Phe

and in combinations. The octapeptide No. 3 gave two peaks (the first is an unidentified impurity) and both the deca- and heptapeptides had the same retention time. The small peaks on the chromatograms, except the two relatively large peaks which elute before 10 min and are contaminants of the octapeptide No. 8, are artifacts and do not represent impurities present in the sample mixture. The results show that such closely related peptides can rapidly be analyzed by this technique. The relative retention values are in agreement with the relative hydrophobicities calculated from the Σf values in Table I, with the exception of the highly non-polar hexapeptide No. 5 which elutes

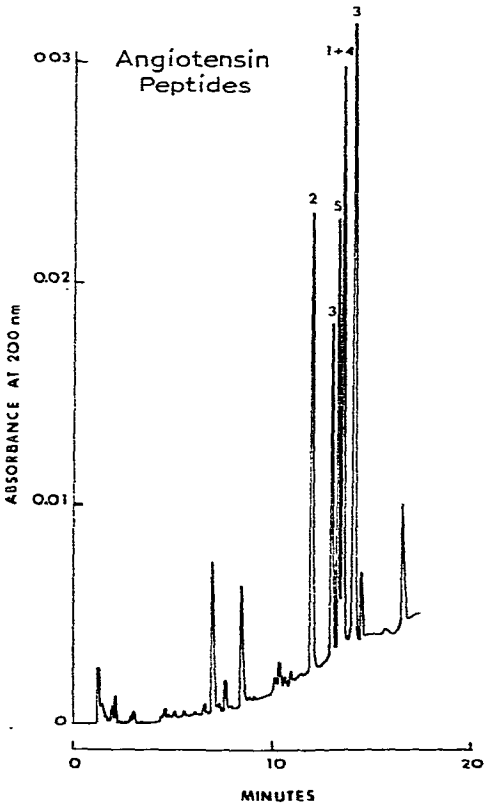

Fig. 10. Separation of angiotensins. Peak numbers, see Table II. The chromatographic conditions were the same as in Fig. 9, except gradient type C (*cf.* Fig. 1) was used.

faster than expected. Obviously the size of the molecule, besides the hydrophobicity of the side chains, also affects the retention.

The tridecapeptide α-melanotropin (porcine α-MSH) has the following amino acid sequence CH_3CO-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. The substance which has been purified by conventional methods gives four peaks under the chromatographic conditions shown in Fig. 11. Further investigation is needed to establish the nature of the individual peaks. They may represent conformers of the peptide whose structure is given above. It is possible, however, that the deacylated peptides and/or a derivative, which oxidized to form methionine sulfoxide, is also present in the "pure" peptide. Each of the four peaks represent a peptide containing tryptophan as established by tandem monitoring of the effluent with the fluorescence detector under conditions where only tryptophan shows appreciable fluorescence.

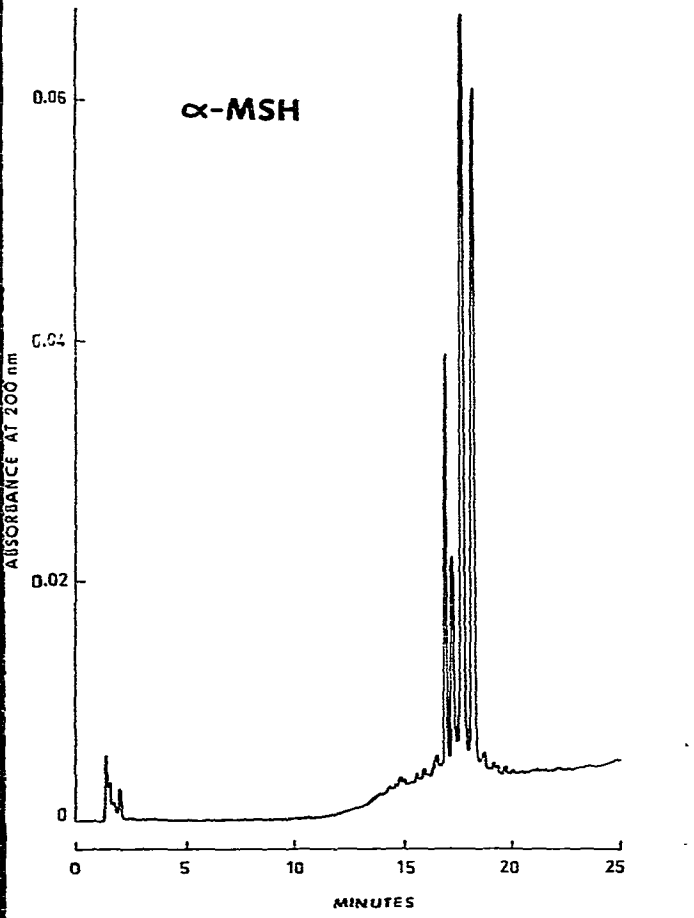

Fig. 11. Chromatogram obtained with melanotropin. The chromatographic conditions were the same as in Fig. 9.

Separation of components of reaction mixtures

Enzymic degradation is frequently employed in determining the sequence of polypeptides. The present technique has been employed to separate the products arising from enzymic degradation of ribonuclease S peptide. This polypeptide, which is obtained by the controlled treatment of ribonuclease A with subtilisin[25] has the following amino acid sequence: Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala. The chromatograms A and B in Fig. 12 show the separation of peptides formed by tryptic[26] and peptic[27] digest of the icosapeptide. Further work is required for the identification of the individual peaks which represent various fragments of the S peptide. The results suggest that, owing to its speed and efficiency, this chromatographic method can be a valuable tool to study the enzymic degradation of polypeptides and proteins.

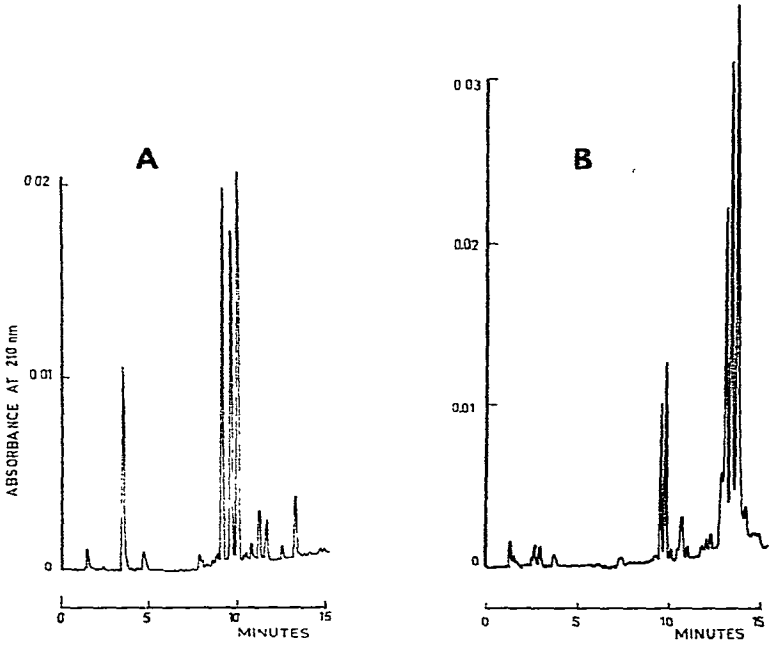

Fig. 12. Chromatograms of tryptic (A) and peptic (B) digest of ribonuclease S peptide. Chromatographic conditions were the same as in Fig. 10, except gradient type E (*cf.* Fig. 1) was employed.

The chromatogram in Fig. 13 shows the components present in the product of a reaction used for the chemical modification of peptide hormones. A fragment (I) of the melanocyte-stimulating hormone, β-MSH, has been reacted with Bolton-Hunter reagent (II) in order to introduce a *p*-oxyphenylpropionic acid moiety[28], which can subsequently be iodinated in order to obtain a ^{126}I-labeled peptide.

His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp
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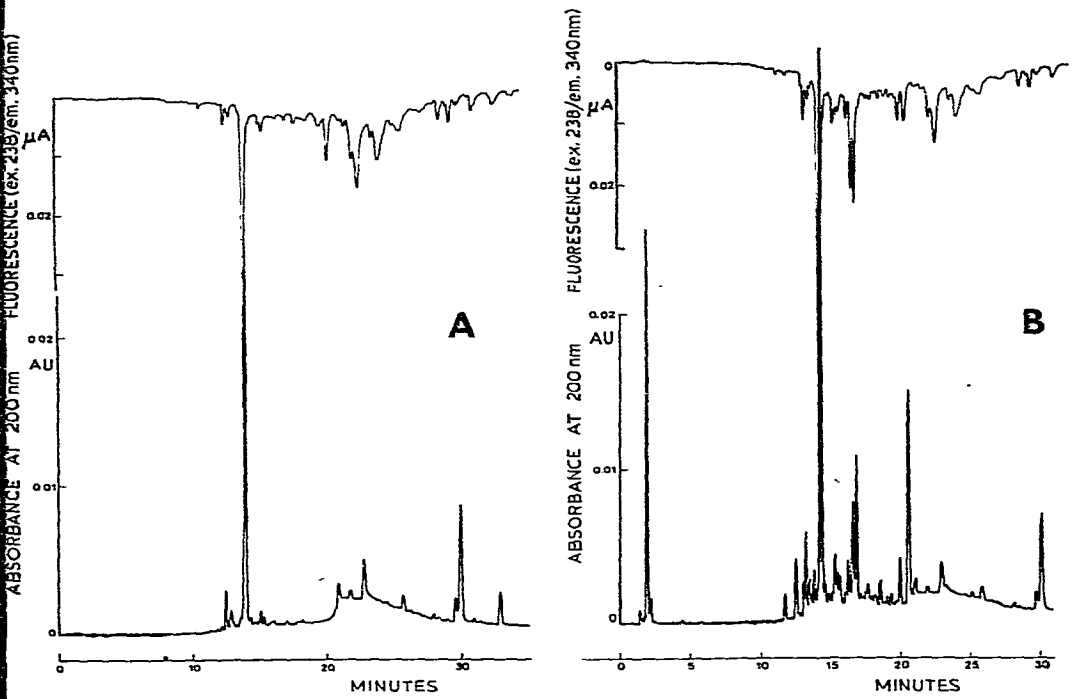
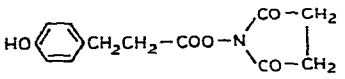


Fig. 13. Chromatograms of a β-MSH fragment (A), whose structure is shown in the text, and its N^{α}-hydroxyphenylpropionyl conjugate. Conditions are given in Fig. 10.



II

The chromatogram of the unreacted β-MSH fragment is shown in Fig. 13A. The product of the Bolton–Hunter reaction was first purified on a conventional cation-exchange resin column; then some unreacted I was added as a marker. A chromatogram of this mixture is shown in Fig. 13B. The largest peak represents the added starting material I and the product of the reaction is believed to elute at 16–17 min. Both the starting material and the reaction products contain tryptophan, which strongly fluoresces so that from the chromatograms at the top, inferences can be drawn as to the elution time of I and its derivatives. The large peak having a retention time of 20 min has been identified as benzene, which was used in the reaction mixture and has not been completely removed in the purification step. No unreacted II, which would have a retention time of 10 min under the conditions employed, was found in the mixture. The remaining large number of peaks that appear on the chromatograms have not been identified. In part at least, they may represent trace contaminants which have been leached out from the polystyrene-type cation-exchange resin used for the purification of the product.

Separation of hydrophilic amino acids

As shown before, the retention of the most polar amino acids and peptides is

insufficient under the conditions employed to obtain adequate resolution to make the technique applicable to the analysis of protein hydrolyzates. The use of carbonaceous stationary phases, *e.g.*, such as introduced by Colin and Guiochon[29], or that of a hydrocarbonaceous bonded phase of high carbon content and low column temperature might give sufficiently high capacity factors for the hydrophilic amino acids and peptides.

An alternative approach is to add an ionic surfactant to the eluent and carry out the separation by a technique often referred to as "ion-pair" or "soap" chromatography[30]. The mechanism underlying this type of chromatography has recently been investigated in our laboratory[31]. On the basis of extensive experimental data, we concluded that in most practical situations the enhancement of the capacity factor of charged eluites is due to ion-pair formation with the surfactant counterion in the mobile phase and the higher capacity factor of the ion-pair complex.

Amino acids can carry positive or negative charges or both. Consequently, there is a great degree of flexibility in designing an eluent with respect to pH and the chemical nature of the ionic surfactant to achieve the separation of amino acids on non-polar stationary phases. In preliminary studies we have used decyl sulfate as the surfactant at pH 2.1, and the separation of some hydrophilic amino acids by this technique is shown in Fig. 14. The results suggest that upon finding a suitable complexing agent and optimizing the pH of the eluent, this approach could lead to the development of a rapid method for amino acid analysis on non-polar stationary phases by HPLC. The sensitivity of the analysis can be enhanced by the use of a postcolumn reactor to form fluorescent derivatives[32].

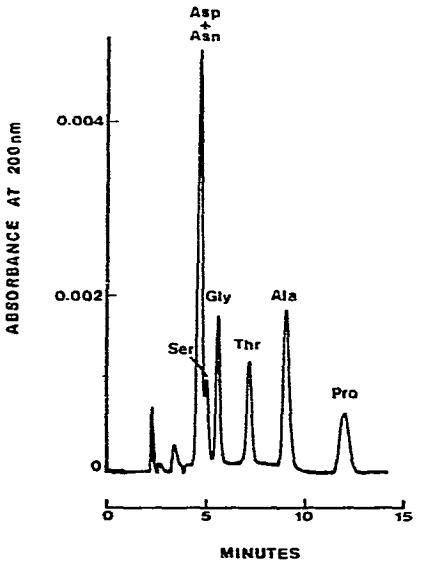


Fig. 14. Separation of hydrophilic amino acids on octadecyl-silica column with decyl sulfate in the neat aqueous eluent. Column: 5-μm LiChrosorb RP-18; eluent, 0.1 *M* phosphate buffer, pH 2.1, containing $3 \cdot 10^{-3}$ *M* decyl sulfate; temperature, 70°; flow-rate, 2 ml/min; ΔP, 150 atm.

CONCLUSIONS

This study clearly demonstrates that HPLC with non-polar stationary phases can be used for the rapid and efficient analysis of peptides. This technique appears to be superior to chromatography on ion-exchanger columns which have conventionally been used for peptide separation. Owing to the diverse architecture and zwitterionic nature of the amino acid and peptide molecules, it is likely that their separation can be accomplished on non-polar stationary phases under a variety of conditions with respect to the pH, the buffer and organic solvent composition of the eluent as well as to the column temperature. Consequently, low eluent pH and high column temperature may represent only one useful set of chromatographic conditions. Gradient elution may not be required for the separation of peptides having similar "hydrophobicities". Nevertheless, it has extensively been used in this investigation when the composition of the mixture was not known or when the solutes had wide ranging hydrophobicities.

As illustrated, the method can be used for conveniently measuring the purity of peptides. Thus, it facilitates the monitoring of reactions for the synthesis and chemical modification of peptides. The technique also allows a rapid separation of the degradation products of polypeptides and proteins. It has a potential as an additional physico-chemical tool in the study of the structure and conformation of peptides. With appropriate sample preparation and a suitable detection system the method could be readily adapted to analyze peptide hormones in biological samples.

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