RAPID ANALYSIS OF PEPTIDE MIXTURES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH NONPOLAR STATIONARY PHASES

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Charcoal, a nonpolar sorbent, had been widely used for the separation of peptides[1] before the advent of ion-exchange chromatography.[2] Recent developments in high performance liquid chromatography revived the interest in the use of nonpolar stationary phases for the separation of biological substances[3,4] by "reversed phase" chromatography, which employs columns packed with 5 or 10-μm porous silica particles having hydrocarbonaceous functions covalently bound to the surface.

This report illustrates the potential of this type of chromatography for the rapid analysis of minute quantities of peptide mixtures. The results suggest that octadecyl-silica columns can be used for fast separation of a wide variety of peptides. By monitoring the column effluent with a UV-detector at 200 nm, the sample components can be analyzed at the subnanomole level without the formation of UV absorbing or fluorescent derivatives.

Experimental

A Perkin-Elmer (Norwalk, Conn.) Model 601 liquid chromatograph with Schoeffel (Westwood, N. J.) FS 770 UV and FS 970 fluorescence detector was used in the gradient elution mode.[5] The sample was introduced with an injection syringe (ES Industries, Marlton, N. J.). The Lichrosorb RP-18, 5-μm columns were supplied by Rainin (Brighton, Mass.). Typical operating conditions are given in the figure legends.

In most experiments 0.1 *M* phosphate buffer, pH 2.1 or 0.5 *M* perchloric acid, pH 0.2 was used as the starting eluent and acetonitrile (Burdick and Jackson, Muskegon, Mich.) as the gradient former. Isocratic elution with the above phosphate buffer was used for the separation of hydrophilic amino acids. The amino acids and peptides were purchased from Sigma (St. Louis, Mo.).

Results and Discussion

In "reversed phase" chromatography solute retention is governed by hydrophobic interactions between the solute and the hydrocarbonaceous ligands at the stationary phase surface. The theoretical basis of this type of chromatography has been analyzed in recent publications.[4,6,7] The increase in the nonpolar

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moiety of the solute molecule or in the surface tension of the eluent augment solute retention. Conversely, a reduction in the effective surface tension of the eluent or the introduction of polar functions, which can strongly interact with the eluent, into the solute molecules result in a decrease in chromatographic retention.

Hydrophobic amino acids and small peptides can conveniently be separated on presently available nonpolar stationary phases as shown in Fig. 1. Other peptides with hydrophobic residues such as Trp, Leu, Ile, Tyr, or Val can also be readily separated under such conditions.

The technique appears to be generally applicable to the analysis of larger peptides, which usually contain some hydrophobic residues. Fig. 2 illustrates the rapid separation of the tryptic digest of ribonuclease S peptide. The chromatograms in Fig. 3 were obtained with porcine α-melanotropin, which has been purified by conventional methods.[8] The comparison of the two superimposed chromatograms shows that each component contains tryptophan. Only tryptophan has appreciable fluorescence under the conditions employed in this study. Some of the peaks may represent deacetylated or oxidized forms of α-MSH and its conformers.

The conditions described in Figs. 1-3 are not suitable for the chromatographic separation of hydrophilic amino acids and small peptides because of the insufficient retention. However the retention of charged species can be enhanced by adding a counterion having a hydrophobic moiety to the eluent. This technique, using an ionic surfactant, is referred to as "ion-pair" or "soap" chromatography.[9] Figure 4 illustrates that with decyl sulfate in the eluent, the hydrophilic amino acids can be separated.

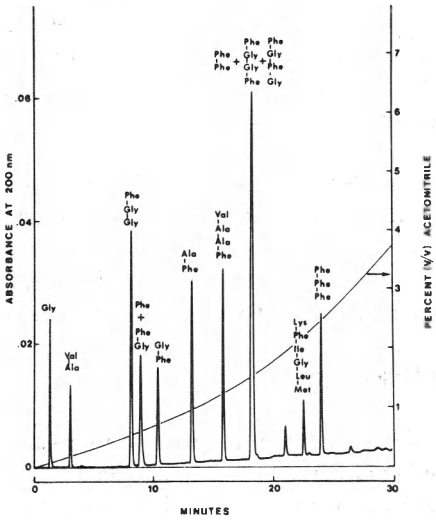

Fig. 1. Chromatogram of small peptides containing hydrophobic amino acids. Column, 5-μm octadecyl-silica, 4.6 mm i.d., 25 cm long; 70°C; flow rate, 2.0 ml/min; inlet pressure, 160 atm; starting eluent, 0.5 *M* perchloric acid, pH 0.2; gradient former, acetonitrile concave gradient in 50 min to 100% at setting 0.3, as illustrated. Sample size approximately 200 ng of each component.

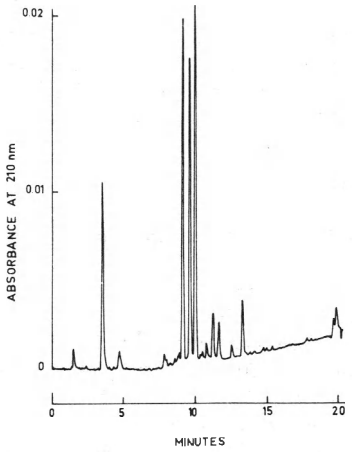

Fig. 2. Chromatogram of the tryptic digest of ribonuclease S-peptide (Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala). Column and operating conditions are the same as those in Figure 1.

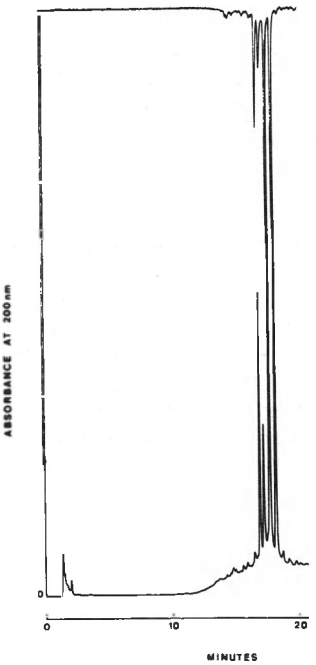

Fig. 3. Chromatogram of porcine α-melanotropin (α-MSH) purified by conventional methods. The amino-acid sequence of the peptide is as follows: CH_3CO-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. The column and operating conditions are the same as in Fig. 1. The chromatogram at the top has been obtained by tandem monitoring of the effluent with a fluorescence detector. Excitation and emission wavelengths are 280 nm and 340 nm, respectively. Full scale corresponds to a detector output of 0.5 μA at a time constant of 0.5 seconds.

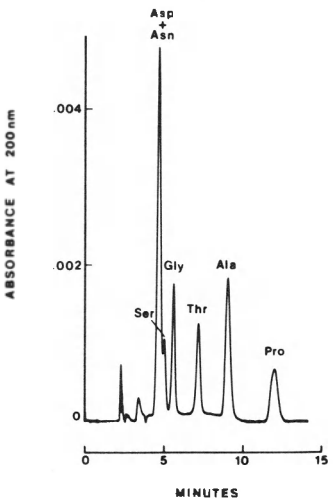

Fig. 4. Chromatogram of polar amino acids on octadecyl-silica with decyl-sulfate in the eluent. Column: 5-μm Lichrosorb ODS, 4.6 mm i.d., 25 cm long; 70°C; inlet pressure, 160 atm; isocratic elution with 0.1 *M* phosphate buffer containing 10^{-3} *M* decyl sulfate; sample size, approx. 100 ng of each component.

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