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Note

Hydrophilic ion-pair reversed-phase chromatography of biogenic peptides prior to immunoassay*

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The determination of biologically relevant peptides in human plasma is difficult, mainly owing to their very low concentrations, the presence of structurally related compounds and the insufficient specificity of the antisera used in radioimmunoassay (RIA) as commonly applied. Consequently, the results for these peptides are correctly defined as "peptide-like immunoreactivities". Gel filtration techniques, sometimes applied to the purification of plasma peptides [1, 2], fail to separate sufficiently peptides of closely related structure. Reversed-phase systems in high-performance liquid chromatography (HPLC) are being used increasingly to separate peptides with high resolution. Molnár and Horváth [3] and O'Hare and Nice [4] reported that adequate resolution is strongly dependent on the pH and ionic strength of the mobile phase. However, the non-volatile mobile phases recommended by these workers render these systems unsuitable for the subsequent determination of immunological or biological activity which is, as yet, the only tool for the quantitation of the minute amounts of peptides in peripheral blood.

We report here about our studies on two reversed-phase systems using hydrophilic ion-pairing reagents with particular respect to their suitability for subsequent immunoassay. The applicability of one system is demonstrated for the chromatographic profiling of adrenocorticotrophin (ACTH) immunoreactivities arising in human plasma.

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EXPERIMENTAL

Instrumentation

A Hewlett-Packard Model 1084B high-performance liquid chromatograph equipped with a variable-wavelength spectrometer was used. Octadecyl-coated silica (LiChrosorb RP-18; Knauer, Berlin, G.F.R.) was used as the stationary phase and stepwise gradient elution was performed. Different eluents were studied as the mobile phase. Eluted HPLC fractions were collected for subsequent RIA in plastic tubes using an UltroRac 7000 collector (LKB, Stockholm, Sweden). A Riedel Model RI 240 gamma spectrometer was used for monitoring ^{125}I radioactivity.

Chemicals

Phenylalanine and tryptophan were obtained from Merck (Darmstadt, G.F.R.). Synthetic human $ACTH_{1-39}$ and $ACTH_{1-24}$ were a gift from Ciba-Geigy (Basle, Switzerland). All other peptides were obtained from Serva (Heidelberg, G.F.R.). Acetonitrile (LiChrosolv grade), ammonium formate and trifluoroacetic acid (TFA) (all analytical-reagent grade) were obtained from Merck. Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.). Charcoal (Norit A) was obtained from Serva.

The following reagents were used for the RIA of ACTH: [^{125}I]-*p*-$ACTH_{1-39}$ and anti-*p*-$ACTH_{1-39}$ were obtained from CEA (Gif-sur-Yvette, France); h-$ACTH_{1-39}$ (Ciba-Geigy) was used as a standard; 0.02 *M* barbital buffer (pH 8.6) containing 1% bovine serum albumin (BSA) was used as the incubation medium.

Methods

For chromatographic studies, polypeptide standards were dissolved in the solvent initiating the gradient run. Depending on the number of aromatic residues within the peptide molecule, amounts between 0.5 and 12 μg were subjected to HPLC. The eluted peptide standards were detected by their UV absorbance at 275 and 254 nm. The flow-rate was 1.3 ml/min throughout. Ammonium formate and trifluoroacetic acid were studied as hydrophilic ion-pairing reagents, and acetonitrile as organic modifier. As the final system, 0.05 *M* TFA and acetonitrile were used for the immunoassay studies.

Peptides were extracted from plasma using a method originally outlined by Bennett et al. [5]. In brief, 4 ml of plasma were passed through a Sep-Pak C_{18} cartridge, then the silica matrix was washed with 5 ml of 0.05 *M* TFA and eluted slowly with 2 ml of acetonitrile—0.05 *M* TFA (80:20, v/v). The eluate was lyophilized, reconstituted with 150 μl of 0.05 *M* TFA and subjected to HPLC. The fractions eluted by HPLC were partly evaporated, then lyophilized and reconstituted in 450 μl of BSA—barbital buffer. Volumes of 200 μl were subjected to RIA in duplicate. The RIA data were evaluated using the "spline approximation" as a standard curve model [6].

RESULTS AND DISCUSSION

The influence of ionic strength and pH on the separation efficiency of

the reversed-phase system is demonstrated in Fig. 1, using tryptophan, phenylalanine and its polymers as the test mixture. Nice and O'Hare [7] emphasized that both acidic conditions and a suitable ionic strength in the primary solvent are essential for efficient reproducible chromatography and that neglect of either results in a marked impairment of peak shape and a decrease in resolution. Our experiments under neutral conditions and at an ammonium formate concentration of 0.05 *M* (Fig. 1b), however, yielded separations equivalent to those obtained under acidic conditions (pH 1.3) and at the same ionic strength (Fig. 1c). Hence, ionic strength is the only prerequisite for effective peptide separations, and the proton concentration may only affect the polar-

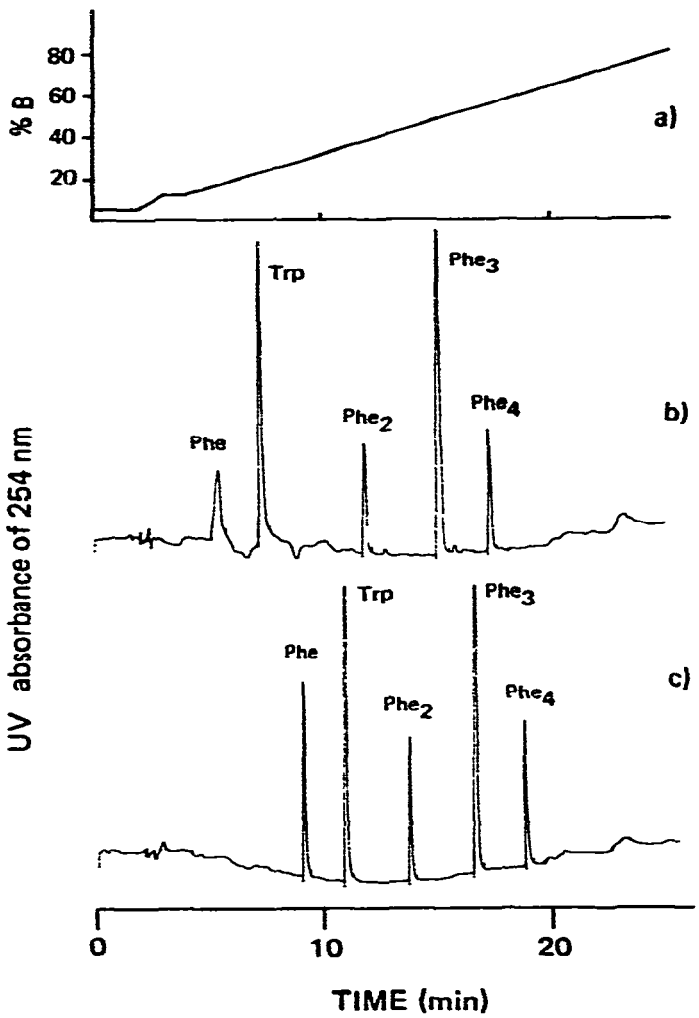

Fig. 1. HPLC of tryptophan (Trp), phenylalanine (Phe) and its polymers (Phe_2—Phe_4). Column, RP-18 (250 × 4.6 mm I.D.) (Knauer); diameter of particles, 5 μm; oven temperature, 40°C; amounts injected, 0.1—0.5 μg; the gradient applied is outlined in (a); attenuation, $6.4 \cdot 10^{-3}$ a.u./cm. The organic modifier was acetonitrile (solvent B). Solvent A in (b), 0.05 *M* ammonium formate; in (c), 0.05 *M* trifluoroacetic acid (pH 1.3).

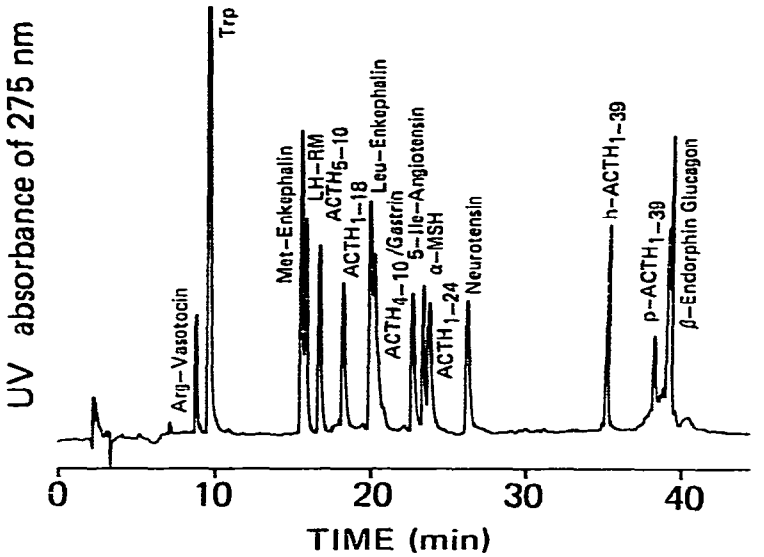

Fig. 2. HPLC of biogenic peptides. Amounts injected ranged from 0.5 to 12 μg. The TFA—acetonitrile system (Fig. 1c) was used. The gradient was increased stepwise from 5% to 80% B within 60 min. Other conditions as in Fig. 1.

ity of the amphoteric amino acids and peptide molecules [8]. Both of the hydrophilic ion-pairing reagents studied [ammonium formate (Fig. 1b) and trifluoroacetic acid (Fig. 1c)] are suitable for subsequent lyophilization and immunological quantitation. As lyophilization of the trifluoroacetic acid phase is more rapid, priority was given to this system, which is based on that outlined by Bennett et al. [5]. Reproducibilities (coefficients of variation) of the retention times ranged from 0.48% for phenylalanine to 0.09% for the tretramer of phenylalanine (n = 12). Such accuracy is essential for measuring peptides that are not detectable by direct classical detection techniques, but by sensitive immunological quantitation in fractions eluted after calibrated retention times.

The efficiency of the system in separating diverse biogenic peptides is demonstrated in Fig. 2. The orders of retention of the peptides are almost completely in agreement with those found by Nice and O'Hare [7] using the non-volatile phosphate system.

The conservation of bio- and immunoactivity of peptides after HPLC was established by assaying ACTH immuno- and bioactivity in fractions eluted by HPLC from a commercially available porcine pituitary ACTH preparation [7]. Owing to the acid and salt conditions used in this system, neutralization of the acid and dilution of the buffer 1:4 was necessary in order to provide immunoassayability of the post-HPLC fractions. Hence, a detection limit of only 200 pg per fraction was achieved, rendering this system unsuitable for monitoring immunoactivities of low-concentration peptides, e.g., ACTH in blood.

The suitability of the present ion-pair reversed-phase system for such purposes is demonstrated in Fig. 3. The volatile nature of the mobile phase provides a detection limit of about 2 pg per fraction. The chromatogram of the

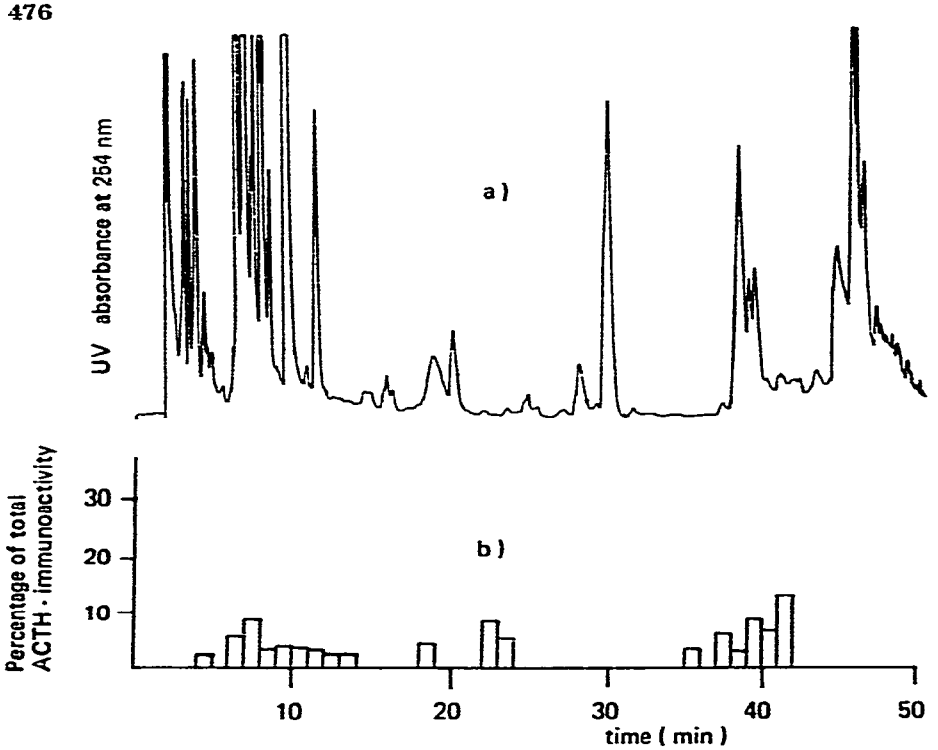

Fig. 3. HPLC of an extract of (a) human plasma and (b) ACTH immunoreactivities arising in the corresponding 1-min fractions. The total amount of ACTH immunoreactivity was 55.5 pg if 200 μl of the total fraction volume (450 μl; see Methods) were radioimmunoassayed. The TFA—acetonitrile system (Fig. 2) was applied.

extract of a normal human plasma sample obtained with UV detection (Fig. 3a) indicates considerable amounts of UV-absorbing materials in plasma that are not attributable to peptides of biological interest. The corresponding chromatogram of ACTH immunoactivities arising in 1-min HPLC fractions (Fig. 3b) displays a considerable variety of ACTH-like compounds, the chemical nature of which is as yet unknown.

In conclusion, the HPLC system described here provides sensitive immunoassayability of low-concentration peptides separated by efficient ion-pair reversed-phase chromatography. It may contribute to the further elucidation of the nature of peptide-immunoactive materials in the human circulation.

REFERENCES

1 R.S. Yalow and S.A. Berson, Biochem. Biophys. Res. Commun., 44 (1971) 439—445.
2 S.J. Ratter, P.J. Lowry, G.M. Besser and L.H. Rees, J. Endocrinol., 85 (1980) 359—369.
3 I. Molnár and C. Horváth, J. Chromatogr., 142 (1977) 623—640.
4 M.J. O'Hare and E.C. Nice, J. Chromatogr., 171 (1979) 209—226.
5 H.P.J. Bennett, A.M. Hudson, C. McMartin and G.E. Purdon, Biochem. J., 168 (1977) 9—13.
6 M. Schöneshöfer, Clin. Chim. Acta, 77 (1977) 101—115.
7 E.C. Nice and M.J. O'Hare, J. Chromatogr., 162 (1979) 401—407.
8 W.S. Hancock, C.A. Bishop, R.L. Prestidge and D.R.K. Harding, Science, 200 (1977) 1168—1170.