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Note

Reversed-phase, ion-pair separation of thyrotropin-releasing hormone and some analogs

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The low molecular weight of the tripeptide thyrotropin-releasing hormone (TRH) has made its unambiguous isolation difficult[1]. Investigation of its biosynthesis has been hampered because of problems encountered in isolating the labelled TRH formed by incubating tissue samples with isotopically labelled precursor amino acids[2]. Because TRH is both polar and of low molecular weight, it is eluted too rapidly from conventional reversed-phase chromatographic systems like those described previously[3,4], to allow its adequate separation from some of its analogs. Evidence has been presented that the formation of ion pairs can facilitate the isolation of the peptide by reversed-phase high-performance liquid chromatography (HPLC)[5]; however, to our knowledge, this technique has not been applied to small, neurally active peptide hormones. By forming an ion pair between the imidazole proton of TRH's histidyl residue and the sulfate of 1-heptanesulfonic acid, a complex is generated which permits an excellent separation of TRH from its analogs.

MATERIALS AND METHODS

A Micromeritics 7000B high-performance liquid chromatograph with a μBondapak C_{18} column (30 cm × 3.9 mm I.D.; Waters Assoc., Milford, Mass., U.S.A.) and a variable wavelength detector (Schoeffel, Westwood, N.J., U.S.A.) were used for all studies. Reagents used were acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), acetic acid (J.T. Baker, Phillipsburg, N.J., U.S.A.), and 1-heptanesulfonic acid (Eastman-Kodak, Rochester, N.Y., U.S.A.). Water was purified with a MILLI-Q system (Millipore, Bedford, Mass., U.S.A.). TRH was a gift of Abbott Laboratories (North Chicago, Ill., U.S.A.); MK-771, a centrally active analog of TRH [L-N-(2-oxopiperidin-6-ylcarbonyl)-1-histidyl-1-thiazolidine-4-carboxamide][6], was a gift of Merck Sharp & Dohme (Westpoint, Pa., U.S.A.); enkephalins were purchased from Pierce (Rockford, Ill., U.S.A.). All other peptides used were purchased from Peninsula Laboratories (San Carlos, Calif., U.S.A.); all peptides contained only L-amino acids.

Peptides (0.2–2.0 μg) were dissolved in 0.1 ml HPLC solvent, as described

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below, and injected into the chromatograph. Since TRH contains neither aromatic residues nor primary amines, it can be monitored only by its peptide bond absorbance at 210 nm. Identification of peaks was confirmed by off-line reaction with the Pauly reagent[7].

RESULTS AND DISCUSSION

Optimal separation of TRH from its analogs was achieved using a solvent system of 6.5% acetonitrile and 0.1% 1-heptanesulfonic acid in 0.02 *N* acetic acid, a flow-rate of 2.0 ml/min and a column temperature of 60°. TRH was eluted in *ca.* 11 min and was distinctly separated from all other TRH analogs tested (Fig. 1). This solvent system is especially useful for TRH-like peptides, since the enkephalins, luteinizing hormone-releasing hormone (LHRH), and even the synthetic tripeptide glutamyl-histidyl-prolineamide are not eluted from the column within a reasonable time period.

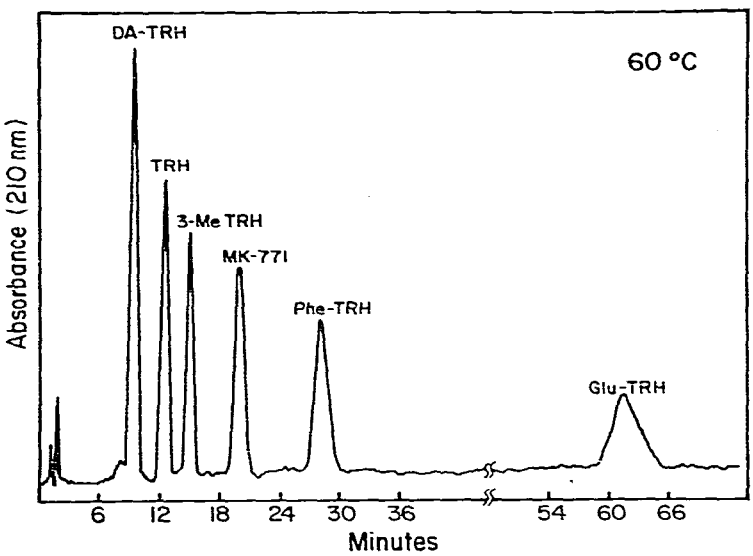

Fig. 1. Typical elution pattern for TRH analogs with 6.5% acetonitrile and 0.1% 1-heptanesulfonic acid in 0.02 *N* acetic acid, with a flow-rate of 2.0 ml/min and a column temperature of 60°. DA-TRH = Pyroglutamyl-histidyl-proline (deamido TRH); 3-MeTRH = pyroglutamyl-3-methyl-histidyl-prolineamide; Phe-TRH = pyroglutamyl-phenylalanyl-prolineamide; Glu-TRH = glutamyl-histidyl-prolineamide.

The importance of using ion-pair formation to separate TRH is shown in Fig. 2. If 1-heptanesulfonic acid is removed from the solvent system, the tripeptides are all eluted rapidly, with very little separation. Glu-TRH with one negative and two positive charges is eluted fastest. When 1-heptanesulfonic acid is included, Glu-TRH can form two ion pairs with its two positive charges and thus is eluted slowest. Pyroglutamyl-phenylalanyl-prolineamide has no charged residues, so its retention time is not affected by 1-heptanesulfonic acid.

While optimal separation is achieved at 60°, a satisfactory, though slower and less sharp, separation can occur at 30° (Fig. 3). If the concentration of acetonitrile is

increased, retention times are markedly shortened and resolution is lost. Similarly, increasing the concentration of acetic acid also diminishes resolution and interferes with absorbance detection at 210 nm. (This problem has been encountered with previously described ion-exchange HPLC purifications of TRH[8].) If the pH is increased by buffering the acetic acid with ammonium acetate, the peaks are broadened and resolution is lost. A simple system, based on acetic acid, was found to give optimum separation (Fig. 1) and also allowed further analysis of column eluant if timed fractions were collected and lyophilized.

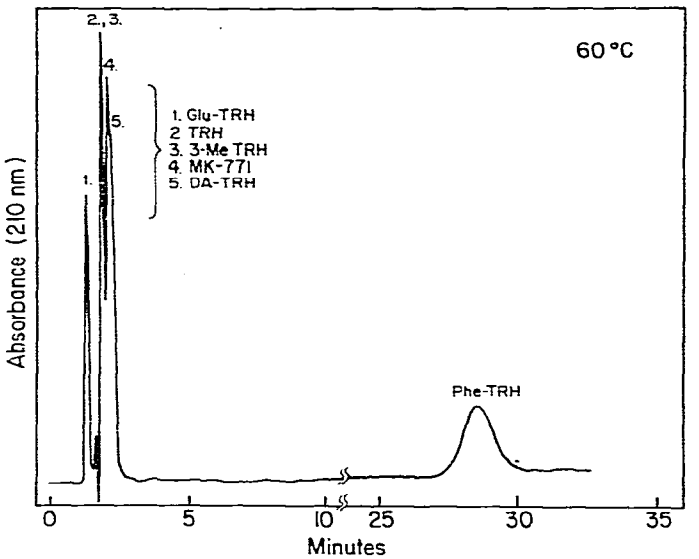

Fig. 2. Elution pattern for TRH analogs obtained as in Fig. 1 except that 1-heptanesulfonic acid has been deleted from the solvent system. Compounds as in Fig. 1.

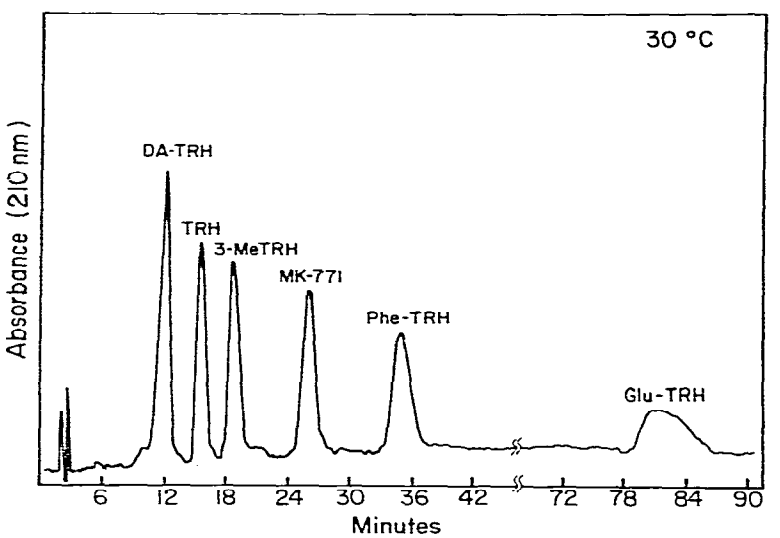

Fig. 3. Elution pattern for TRH analogs obtained as in Fig. 1 except at a column temperature of 30°. Compounds as in Fig. 1.

TRH added to this solvent system can be detected by radioimmunoassay (RIA) if the solvent is lyophilized and then reconstituted in phosphate buffer. Thus a tissue extract can be fractionated by the HPLC and then subjected to TRH immunoassay. This two-step method enhances the specificity of assays of tissue TRH. By increasing the percentage of acetonitrile used, this ion-pairing procedure can also be used for purification of other, larger, neurally active peptides that contain cationic residues.

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