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Note

Isocratic reversed-phase high-performance liquid chromatographic separation of underivatised tyrosine-related peptides of thymopoietin$_{32-36}$ pentapeptide

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Thymopoietin is a immunoregulatory thymic hormone whose 49 amino acid sequence is known[1]. This polypeptide hormone has been shown to influence prothymocyte to thymocyte differentiation[2,3] as well as the functioning of peripheral cells involved in immunity[4,5]. A pentapeptide (TP5), comprising the amino acid residues 32–36 of thymopoietin, was shown to have the biological properties of the parent thymic hormone both *in vitro* and *in vivo*[6]. This pentapeptide (NH_2-Arg-Lys-Asp-Val-Tyr-COOH) is currently under pharmaceutical development and is being evaluated clinically for the treatment of rheumatoid arthritis[7].

Because of the low plasma levels of TP5 anticipated following administration of a therapeutic dose of the pentapeptide, a tritiated peptide ([^{3}H]TP5, radiolabelled on the tyrosine residue) was synthesized to study the *in vitro* stability of the pentapeptide in human plasma as well as the disposition of the drug-related radioactivity in animals[8]. High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) were developed to assay for the [^{3}H]TP5 as well as to serve as a method to purify the labelled peptide from radioactive preparations. The TLC method, used in an *in vitro* stability study, was previously reported[8].

In this communication an HPLC method is described for the separation of underivatised tyrosine-related peptide and amino acid fragments of TP5 (*i.e.*, the pentapeptide, tetrapeptide, tripeptide, dipeptide, and tyrosine). The method utilizes a reversed-phase, ion-paired chromatography similar to that reported by Rivier[9]. Discussion of the pentapeptide stability and the resolution of the reference compounds under various chromatographic conditions is presented.

MATERIALS AND METHODS

Synthesis of the pentapeptide (TP5), tetrapeptide, tripeptide and dipeptide was performed by Peninsula Labs. (San Carlos, CA, U.S.A.). The amino acid L-tyrosine was obtained from Calbiochem (San Diego, CA, U.S.A.). Structures of these compounds are given in Fig. 1.

High-performance liquid chromatographic system

A Model ALC 202/401 HPLC system (Waters Assoc., Milford, MA, U.S.A.) operated at ambient temperatures, equipped with Model M-6000A pumps, a Model

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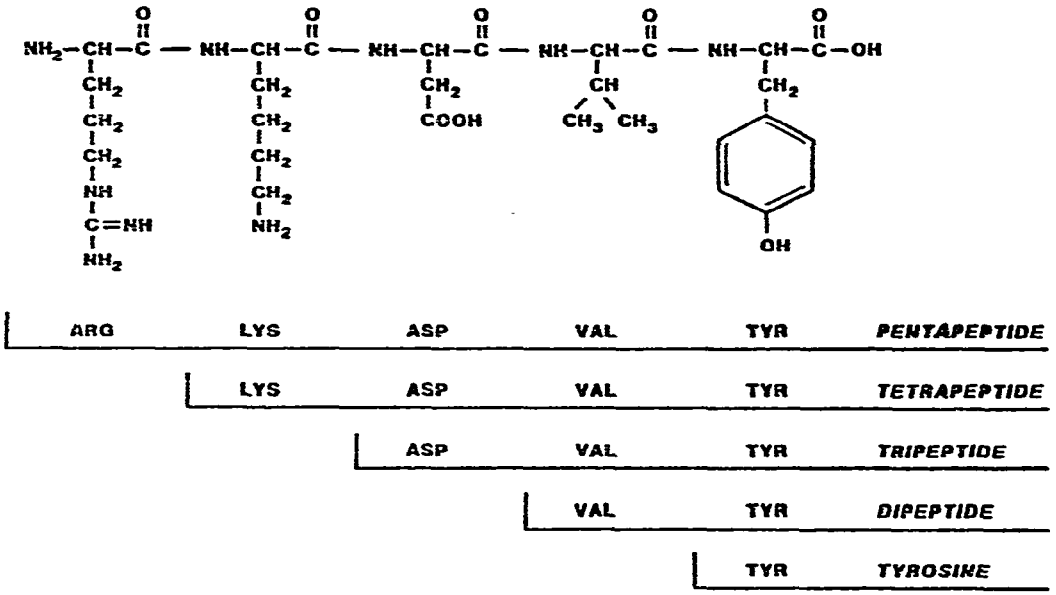

Fig. 1. Structures of the reference compounds.

440 absorbance detector (280 nm) and a U6K septumless injector, was employed. Chromatograms were traced on a dual-pen strip chart recorder (Linear Model 391, Linear Instruments, Irvine, CA, U.S.A.) maintained at a speed of 6 in./h.

Column and solvent system

Separation of all the tyrosine-containing reference compounds was achieved using a 1 ft. × 1/4 in. O.D. stainless-steel column with chemically bonded μBondapak C_{18} reversed-phase support (Waters Assoc.) and an aqueous triethylammonium phosphate (TEAP)–acetonitrile (Burdick and Jackson, Muskegon, MI, U.S.A.) (96:4, v/v) mobile phase, 1 ml/min flow-rate. The TEAP buffers (0.13–0.002 *M* phosphate) were prepared by diluting phosphoric acid (85%) with distilled water to give the appropriate molarity of phosphate. The pH of the solution was then adjusted by addition of triethylamine (reagent grade, Mallinkrodt, St. Louis, MO, U.S.A.). Retention times of the reference compounds were determined with the Model 3352B computer (Hewlett-Packard, Avondale, PA, U.S.A.), and quantitation of TP5 was achieved using peak-area integration performed by the same computer.

Stability of the pentapeptide under the conditions used to chromatograph the compound was evaluated by recycling the TP5 peak eluting from the column. The resulting chromatograms of the recycled TP5 peaks were then examined for signs of instability, such as shouldering or the appearance of secondary peaks.

Calibration curves were generated which compared detector response (computer integration peak areas) to the amount of TP5 applied to the HPLC system (microlitres of a stock 1 mg/ml TP5 aqueous solution).

RESULTS AND DISCUSSION

HPLC separations of peptides based on ion exchange, gel permeation, reversed-phase and affinity chromatographies have been reviewed[10]. The isocratic HPLC method described in this report for the separation of underivatised tyrosine-related compounds of TP5 was developed for use in the analysis and purification of radiolabeled TP5 (tritiated in the tyrosine residue). The method employs reversed-phase paired-ion methodology reported by Rivier[9]. Specifically, a μBondapak C_{18}

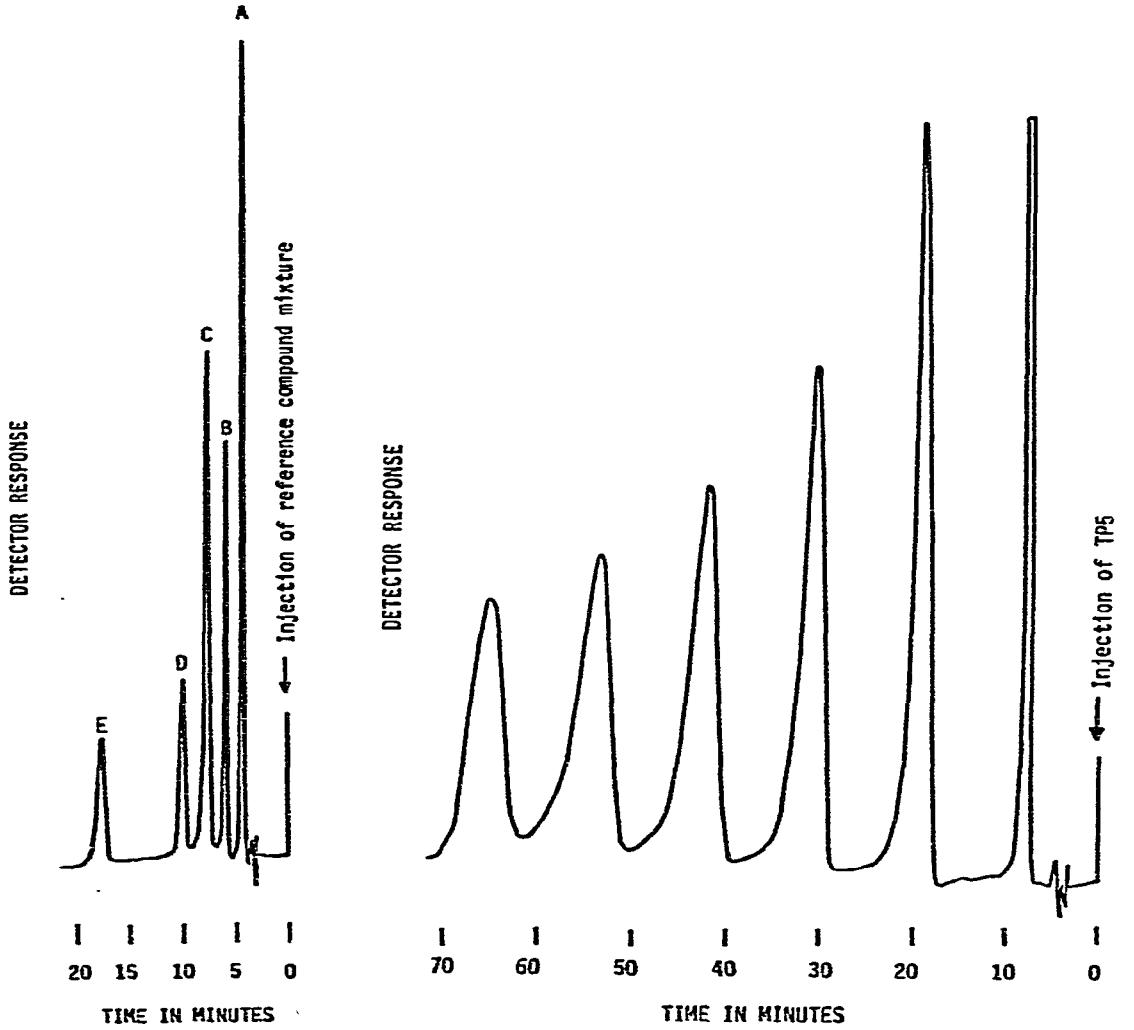

Fig. 2. Detector response *versus* time. HPLC method (μBondapak C_{18} reversed-phase support with 0.08 *M* TEAP (pH 4.0)–acetonitrile, (96:4 v/v) mobile phase) where the pentapeptide, tetrapeptide, tripeptide, dipeptide and tyrosine are peaks C, D, E, B and A, respectively.

Fig. 3. Detector response *versus* time. Chromatogram of HPLC method (μBondapak C_{18} reversed-phase support with 0.08 *M* TEAP (pH 4.0)–acetonitrile (96:4, v/v) mobile phase) recycling eluate containing TP5.

reversed-phase support and a triethylammonium phosphate (TEAP) buffer–acetonitrile mixture (96:4, v/v) as the mobile phase were employed.

Separation of reference compounds

Resolution of the pentapeptide, tetrapeptide, tripeptide, dipeptide and tyrosine was obtained with several different molar phosphate buffer concentrations. A representative separation is shown in Fig. 2 (0.08 *M* TEAP, pH 4.0–acetonitrile (96:4 v/v) mobile phase and μBondapak C_{18} reversed-phase support). The retention times (in minutes) of the pentapeptide, tetrapeptide, tripeptide, dipeptide and tyrosine were 7.5, 9.8, 17.5, 5.9 and 4.3, respectively. Similar chromatograms were generated with the 0.03 *M* and 0.02 *M* TEAP (pH 4.0) buffer systems. The remaining pH 4.0 phosphate buffers (*i.e.* the 0.13 *M* and 0.002 *M* TEAP) did not adequately separate the

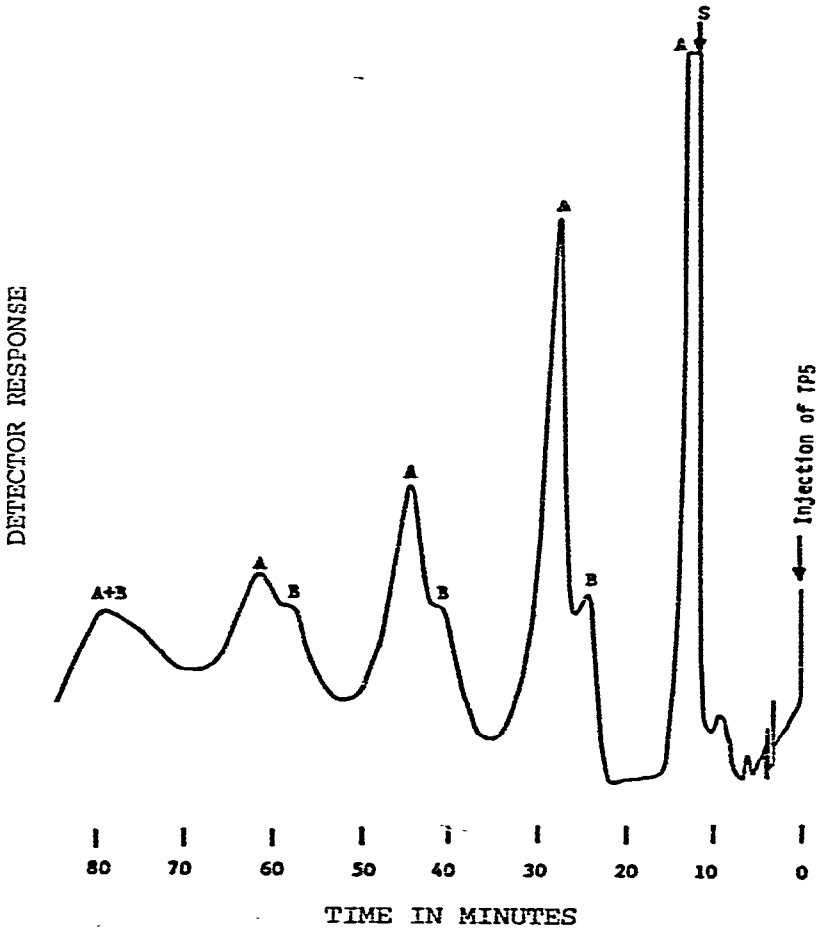

Fig. 4. Detector response *versus* time. Chromatogram represents the recycling of eluate containing TP5 (peak A) using HPLC method, μBondapak C_{18} reversed-phase support with 0.02 *M* TEAP (pH 6.7)–acetonitrile (96:4, v/v) mobile phase. Peak B is "apparent degradation product" and S indicates start of recycling mode of HPLC.

reference compounds. The elution sequence, however, was maintained in the pH 4.0 TEAP mobile phase systems over the entire molarity range investigated (0.13 *M* to 0.002 *M* phosphate): tyrosine (first), dipeptide, pentapeptide, tetrapeptide and tripeptide (last).

Stability

The procedure of recycling the HPLC eluate containing the compound of interest to evaluate the stability of the compound under chromatographic conditions[11] was employed in this study. The stability of the pentapeptide at pH 4.0 (0.13 *M* to 0.002 *M* TEAP) buffer conditions was demonstrated by the lack of secondary peaks of shouldering appearing in the chromatograms upon recycling the eluate

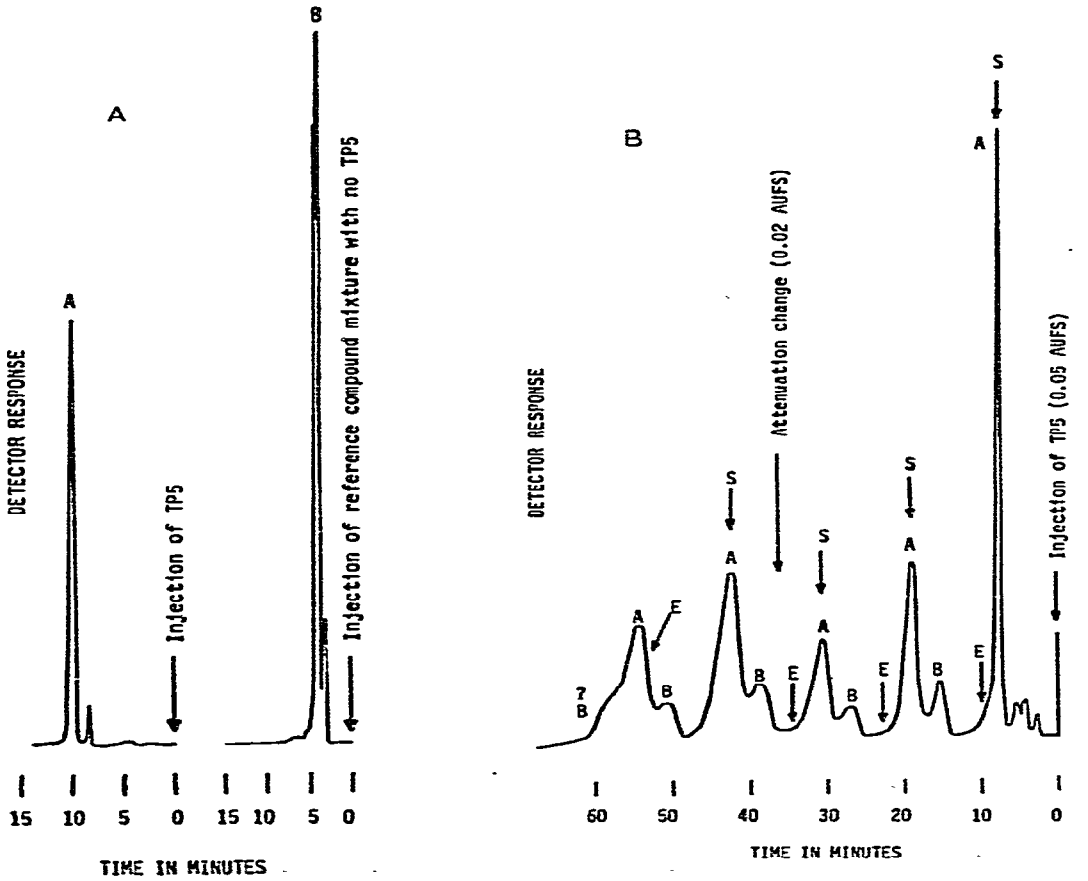

Fig. 5. A, Detector response *versus* time where peak A represents TP5 (15 μg) and peak B represents a mixture of the tetrapeptide, tripeptide, dipeptide and tyrosine using a second HPLC system (0.005 *M* ammonium acetate, pH 6.7, mobile phase and a μBondapak CN reversed-phase support). B, Detector response *versus* time (HPLC method: μBondapak CN reversed-phase support with 0.005 *M* ammonium acetate, pH 6.7, mobile phase). Chromatogram represents recycling of eluate containing TP5 (peak A), where B is "apparent degredation product(s)" of TP5, S indicates start of recycle mode and E indicates end of recycle mode of HPLC.

containing TP5 (Fig. 3 illustrates a representative chromatogram of a TP5 recycle run).

Interestingly, other HPLC systems, where adequate separation of all the tyrosine-related compounds was not demonstrated, exhibited conditions in which TP5 appeared unstable. For example, under 0.02 *M* and 0.002 *M* TEAP buffer (but not at 0.13 *M*, 0.08 *M* or 0.03 *M* TEAP) pH 6.7–acetonitrile (96:4, v/v) and C_{18} reversed-phase conditions, obvious shoulder patterns of the TP5 peak and secondary peaks were generated upon recycling the HPLC eluate containing the pentapeptide (see Fig. 4). Also, a similar phenomenon was exhibited with a μBondapak CN reversed-phase support and a 0.005 *M* ammonium acetate (pH 6.7) mobile phase HPLC system (see Fig. 5B) in which TP5 was adequately separated from the other unresolved reference compounds (Fig. 5A). This shouldering and secondary peak formation observed upon recycling the HPLC eluate containing TP5 suggest instability of the pentapeptide under low ionic strength and/or neutral pH chromatographic conditions. Alternatively, a mixture of retention mechanisms, as suggested by Hancock *et al.*[12], such that simulataneous action of two retention mechanisms, ion exchange and ion pair formation, may be causing the multiple peaks for a single compound upon recycling. In addition, the appearance of multiple peaks has been interpreted as being

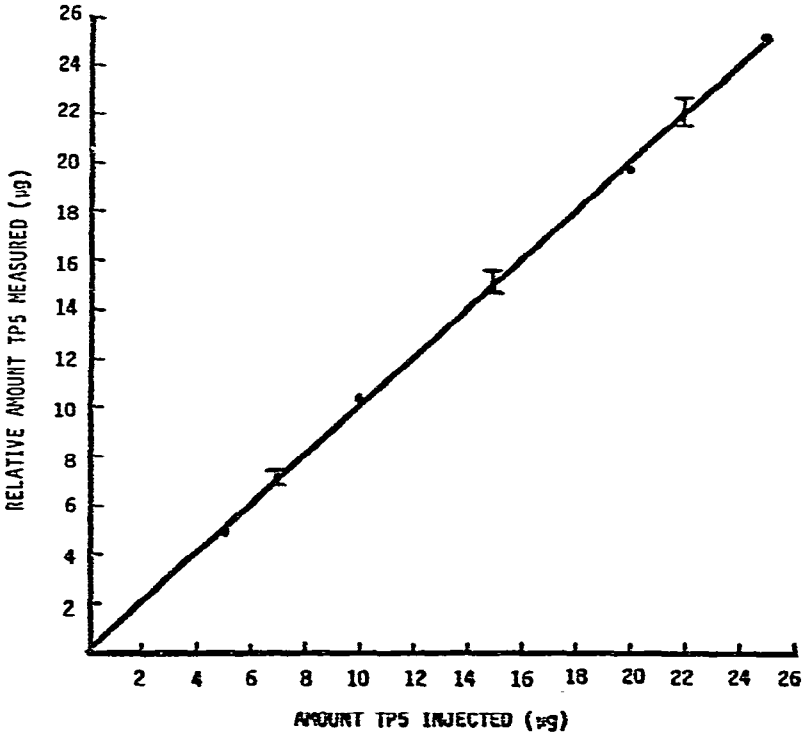

Fig. 6. Pentapeptide calibration curve (computer integrated peak area in relative micrograms measured *versus* micrograms of pentapeptide injected) generated at 0.08 *M* TEAP (pH 4.0)–acetonitrile (96:4, v/v) on a μBondapak C_{18} support. Linear regression line is $y = 0.996x + 0.226$ with a 0.9990 correlation coefficient.

a consequence of polymeric complexes formed by ionic or hydrogen bonds of the peptides (or amino acids)[13]. Whatever the reason for the multiple peak phenomenon, improved chromatographic performance regarding precision and accuracy was attained by increasing the ionic strength and/or decreasing the pH of the mobile phase.

Linearity

A calibration curve for TP5, with 0.08 *M* TEAP (pH 4.0)–acetonitrile (96:4, v/v) used as the mobile phase, is shown in Fig. 6. (*N.B.* $n = 1$ for 5, 10, 20 and 25 μl injections; $n = 5$ for 7 and 15 μl injections; and $n = 3$ for 22 μl injection.) Linearity was demonstrated from 5 to 25 μg TP5 with a correlation coefficient of 0.9990 and a calculated linear regression of $y = 0.996x + 0.226$.

CONCLUSION

An HPLC method is described for the separation of the related tyrosine-containing compounds of TP5. The method employs reversed-phase paired-ion chromatography under isocratic conditions. Linearity of sample recovery over the range 5–25 μg of TP5 was demonstrated by monitoring at 280 nm. In addition, increasing the ionic strength and/or decreasing the pH of the mobile phase improved the stability of the pentapeptide under the chromatographic conditions employed.

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