Journal of Chromatography, 444 (1988) 345–348
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1285

REVERSED-PHASE PREPARATIVE CHROMATOGRAPHY OF [D-Ala^1]-PEPTIDE T AMIDE

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SUMMARY

[D-Ala^1]peptide T amide is a metabolically stable and more potent analogue of peptide T, a proposed inhibitor of human immunodeficiency viral infectivity of human T-cell lymphocytes. The peptide was synthesized by solid-phase methods to provide amounts of several grams. The product was purified by chromatography on a 25 cm × 2 in. column of DuPont Zorbax Pro-10 C_8 (10 μm) packing. Sample loads of 100–450 mg were chromatographed isocratically in 0.1% trifluoroacetic acid and 5% acetonitrile at a flow-rate of 110 ml/min. Under these conditions, the pure peptide fraction was eluted reproducibly between 15 and 22 min. After solvent removal and lyophilization, the recovery of pure peptide was 50% by weight.

INTRODUCTION

An octapeptide fragment of the human immunodeficiency viral (HIV) envelope glycoprotein has been shown to prevent HIV infectivity of human T-cell lymphocytes[1]. This peptide, called peptide T due to the presence of four threonine residues in the sequence, has stimulated development of potential AIDS (acquired immune deficiency syndrome) therapeutics derived from its structure. An analogue, [D-Ala^1]peptide T amide, was found to be more potent in the *in vitro* cell infectivity assay[1]. The peptide was synthesized by solid-phase methods and part was purified by counter-current chromatography as described[2]. In this report we are describing the reversed-phase preparative chromatography of the synthetic peptide.

It is often necessary to use high-resolution reversed-phase packings to separate impurities common in synthetic peptide products that are eluted close to them. To produce several grams of pure peptide it is important to work with columns larger in diameter and to use flow-rates higher than those used in analytical chromatography in order to preserve the linear velocity and reproduce the separation achieved

0021-9673/88/$03.50

in the latter. Therefore, for maximum resolution the same 10-μm packing material was used as in the analytical column but in a 2-in. diameter column. We recently provided 3 g of D-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr-NH_2 for pre-clinical studies (to C. B. Pert and M. R.Ruff of the National Institute of Mental Health).

EXPERIMENTAL

Materials

All solvents and reagents were HPLC- or reagent-grade respectively. Distilled water was passed through a Barnstead Nanopure II cartridge purification system. [D-Ala[1]]peptide T amide was synthesized manually by solid-phase methods[3] on 10 g of *p*-methylbenzhydrylamine resin. The butyloxycarbonyl-protected amino acid derivatives were coupled in sequence as dicycloheylcarbodiimide and hydroxybenzotriazole-generated active esters. The peptide resin that resulted (20.7 g) was treated with hydrogen fluoride in four 5-g batches to give 7.3 g of crude synthetic peptide[2].

Analytical chromatography

The peptide was chromatographed analytically in order to characterize and separate the impurities and to develop conditions for use in a wider diameter column of the same packing material. Amounts of 100–200 μg of the peptide were chromatographed at 0.8 ml/min or 1 ml/min on a 25 × 0.46 cm column of DuPont Zorbax Pro-10 C_8 (10 μm) in 0.1% aqueous trifluoroacetic acid with a gradient of acetonitrile from 5% to 20% over 20 min (Fig. 1). The instrumentation used for analytical chromatography was from Waters/Millipore consisting of two Model 510 extended flow pumps, a Model 680 gradient controller, U6K injector, Model 481 variable-wavelength UV detector and a BBC Goerz-Metrawatt SE 120 recorder. The aqueous solvent was filtered through a 0.45-μm filter and degassed under vacuum.

Preparative chromatography

For preparative chromatography, a solution of peptide (10 mg/ml water) was filtered through a glass fiber filter (Whatman, 934-AH). The separation was carried out in a 25 cm × 2 in. steel column packed with DuPont Zorbax Pro-10 C_8 (10 μm) material. Preparative chromatography was carried out with two instruments. The first, a Varex PSLC-100 chromatograph, had three solvent inlets, a diaphragm pump that provided flow to 180 ml at a back pressure of 5000 p.s.i., five outlet collection valves, a variable-wavelength absorbance detector with a 35-μl flow cell and a computerized gradient control. Sample was loaded automatically with a 10-ml syringe. In these experiments the amount of peptide loaded was limited by the concentration of the peptide solution (usually 10–20 mg/ml).

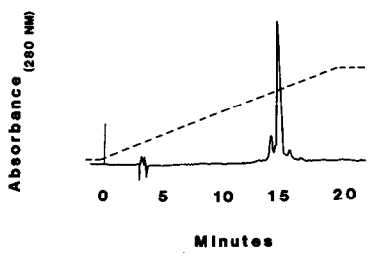
The second instrument, a bench-top Varex VERSA Prep, consisted of a dual head piston pump with flow-rates up to 200 ml/min and 4000 p.s.i. and sample loading via an analytical pump. Additional components consisted of a semi-prep flow cell in a variable-wavelength UV detector and fifteen automatically selected outlet fraction collection valves. This instrument could be used in both analytical and preparative modes. As with the PSLC-100, gradient control was computerized, and high-pressure switching valves were operated by compressed air at 80 p.s.i. Larger volumes of the sample solution could be loaded onto the column by this instrument.

The chromatograph conditions utilized in both instruments after sample loading were isocratic elution with 0.1% aq. trifluoroacetic acid and 5% acetonitrile at a flow-rate of 110 ml/min. After elution of the sample, another injection was made within 10 min. Usually, at the end of a day of multiple runs, the column was washed with 100% acetonitrile. During initial trial runs the two main fractions were analytically chromatographed to determine the content of pure peptide. In ensuing runs, the major peak determined to contain pure peptide was collected, and the solvent was removed at 50°C in a rotary flash evaporator. The resulting residue was lyophilized from water to yield a white powder. The peptide was analyzed by high-performance liquid chromatography (HPLC) and amino acid analysis of a 6 *M* hydrochloric acid, 22-h, hydrolysate gave expected molar ratios of the amino acids: Asp, 1.05; Thr, 3.93; Ser, 1.14; Ala, 1.20; Tyr, 0.67.

RESULTS AND DISCUSSION

Crude peptide appeared to be 85% pure, as assessed by analytical HPLC (Fig. 1). In chromatography on the Zorbax analytical column, a gradient was found to be necessary to elute the peptide. However, for convenience, isocratic conditions were first tried on the preparative column and they appeared to work. A concentration of 5% acetonitrile was found adequate for eluting the compound. These conditions were adopted for the remainder of the work. Initial runs were performed on the PSLC-100. Chromatography of 200 mg gave results very similar to the analytical separation. The minor impurity that is eluted before the major material was separated in the preparative run. The area of the major peak was 86%, similar to the relative peak response in the analytical separation. The major material began to be eluted after *ca.* 15 min and was completely eluted by 22 min. Thus, these conditions at the faster flow-rate preserved the linear velocities and maintained the separation.

The capacity of the column was not exceeded in these experiments. In the VERSA Prep more sample was loaded via the analytical pump (Fig. 2). The largest amount chromatographed was 466 mg, and the separation of closely eluted impurities was maintained at this high sample loading.



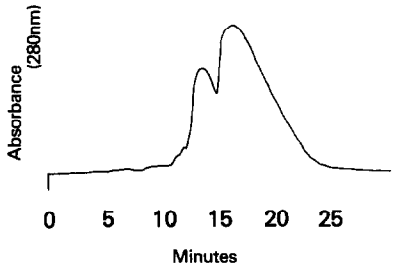


Fig. 1. Analytical chromatography of 200 μg crude [D-Ala¹]-peptide T amide on a Zorbax Pro-10 C_8 (10 μm) column, 25 × 0.46 cm, in 0.1% aq. trifluoroacetic acid with a gradient of acetonitrile (dashed line), 5–20% in 20 min, at a flow-rate of 0.8 ml/min. Detection was at 280 nm with 0.2 units full scale. (Reprinted from ref. 2).

Fig. 2. Chromatography of 240 mg peptide T analogue loaded in 27 ml water on a Zorbax Pro-10 C_8 (10 μm) column, 25 cm × 2 in., in the VERSA Prep instrument using a solvent system of 0.1% aq. trifluoroacetic acid and 5% acetonitrile at a flow of 110 ml/min.

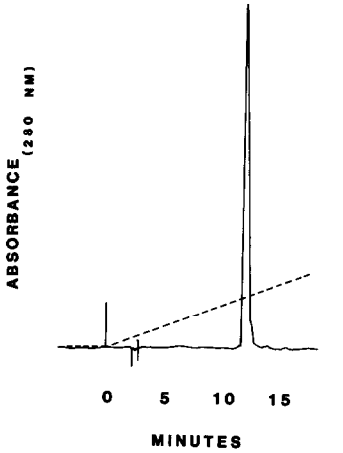


Fig. 3. Analytical chromatography of 100 μg of the material recovered in the major peak of the preparative chromatography of the peptide T analogue. The conditions are the same as described in Fig. 1.

The automatic select valves rendered collection of the pure fraction very easy. In one day, 3 g of peptide was chromatographed in 11 portions. Over 5 g of crude peptide was purified in 32 portions with a yield of 2.6 g of pure product. The entire procedure, including the injection of sample, pump operation, and collection could be automated by the computer control. The results of the preparative purification, shown in Fig. 3, indicate total purification by this method.

Our results show that the equivalent of analytical separation on high-resolution packings can be achieved on a large-diameter column of the same packing. By multiple sample injections large amounts can be processed per day. Our experiment demonstrates successful scale-up from analytical chromatography to preparative chromatography with removal of impurities that are very closely eluted.

ACKNOWLEDGEMENTS

We thank Thomas Finn for helpful discussions and Sara Gluch for amino acid analysis.

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