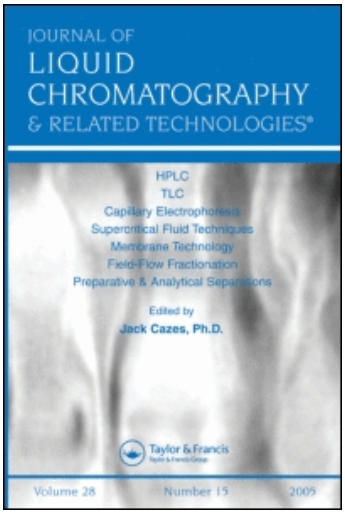

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NEUROENDOCRINE PEPTIDES - ANALYSIS BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high performance liquid chromatographic (HPLC) method is described for determining the biologically active neuroendocrine peptides thyrotropin releasing hormone (TRH), leucine (leu) and methionine (met) enkephalin, angiotensin II, delta sleep inducing peptide (DSIP), luteinizing hormone releasing hormone (LHRH), substance P and growth hormone release inhibiting factor (somatostatin). The selection of mobile phases was limited to these systems that do not exhibit strong absorbtion at 215 nm and 254 nm. Under isocratic conditions at room temperatures with the appropriate selection of mobile phase it was possible by reversed phase chromatography to resolve all of the peptides investigated. We can resolve with the systems employed peptides differing by only one amino acid in chain length as well as peptides differing by only one amino acid in the chain sequence. The method is rapid, does not require derivitization, can be used with aqueous matrixes and is sensitive in the nanomolar range.

Our research has shown that most synthetic peptides lack purity and that all of the peptides except LHRH lack stability when stored in aqueous sterile solution at 8°C for four weeks. The implications of this latter finding are under investigation.

INTRODUCTION

The neuroendocrine peptides have a wide range of physiologic and pharmacologic activities (1, 2, 3). Among the structurally isolated and identified peptides studied were the tripeptides, TRH, the pentapeptides, leu and met enkephalin, the octapeptide, angiotensin II, the nonapeptide, DSIP the decapeptide, LHRH, the nonadecapeptide, substance P and the tetradecapeptide, somatostatin. Analysis of these peptides has relied almost exclusively upon radioimmunoassay, a method that fails to take into account or identify impurities that are inevitable in the synthetic peptides used in antibody formation. Other assay techniques include ion exchange and gel filtration chromatography, electrophoresis and amino acid analysis. However, HPLC may become the method of choice for the rapid analysis of biologically active peptides at the nanomolar quantities. Previously described HPLC chromatographic techniques utilized in the separation of synthetic peptides or naturally occurring peptides used octadecylsilyl silica supports and gradient elution procedures containing such solvents as acetonitrile, ethanol, and methanol with trichloroacetic acid (4, 5, 6). More recently, solvent gradient elution procedures were found to be unnecessary to obtain excellent resolution of peptides (7, 8). The purpose of this research was to demonstrate the usefulness of a reversed phase system of HPLC for the identification and quantification of biologically active peptides.

APPARATUS

A high performance liquid chromatographic system Waters Associates Inc. (Melford, Massachusetts) was used with a M6000-A solvent delivery unit, a U6K universal liquid chromatograph injector coupled through a column to either a Waters model 440 fixed wavelength UV detector or a waters model 450 variable wavelength UV monitor, both having an 8 µl flow through cell, and a Linear

Instrument Corporation (Irvine, California) dual channel chart recorder. This system was assembled from its component parts for this investigation.

The Fatty Acid Analysis^{R} analytic column (3.9 mm I.D. x 300 mm L.) particle size of 10 microns was obtained from Waters Associates, Inc. Precision Sampling Corporation Pressure-Pak liquid syringes series B-110 (Baton Rouge, Louisiana) were used for making sample injections. All solvents and peptide sample solutions were filtered by means of the Waters Associates Solvent Clarification Kit or the Waters Associates Sample Clarification Kit, respectively.

CHEMICALS

All reagents were of analytic grade. Methanol (HPLC) grade was supplied by Burdick and Jackson Laboratories (Muskegon, Michigan). Acetic acid, $NaH\ PO_4 \cdot H_2O$ and $Na_2H\ PO_4$ Fisher Scientific Company, (Pittsburgh, Pennsylvania); and KH_2PO_4 and K_2HPO_4 were supplied by Mallenckrodt Inc. (St. Louis, Missouri). Water deionized and then distilled in glass was further purified by means of the Milli-Q^{R} Millipore Corporation (Bedford, Massachusetts) water purification system to remove impurities and residual ions. This specially purified water was used to make all the aqueous solutions within three days after being purified to minimize contamination.

METHODS

A flow rate of 1.5 ml/min was used in eluting the specific samples. All tests were carried out isocratically at room temperature (21-22°C). Sample sizes and the specific solvent system will be given under each figure. Detection was at 215, 254 or 280 nm, depending on the nature of the sample involved. All peptides were dissolved in treated deionized distilled water and were filtered by means of the sample clarification kit prior to injection. For the mobile phase the filtered solvents were mixed in required volume ratios and equilibrated for at least one hour prior to use.

The columns were solvent conditioned prior to sample injection by passing the mobile phase for 30 minutes through the column at the rate of 1.5 ml/min and where needed additional solvent was passed through the column until the base line was stabilized. When eluting solvent systems were changed, the columns were first washed with 50% MeOH/50% H_2O for 30 minutes at the established flow rate and then conditioned to the new mobile phase in the manner previously described. Upon sample injection, sufficient mobile phase was used for chromatographic development and was then continued until baseline resolution was reestablished. Columns were washed each evening after utilization for a minimum of 30 minutes using a 50% MeOH/50% H_2O at the established flow rate.

PEPTIDES

The synthetic peptides, TRH, leu and met enkephalin, angiotensin II, DSIP, LHRH, substance P and somatostatin were obtained commercially from either Peninsula Laboratories Inc. (San Carlos, California); Beckman Instruments, Inc. (Palo Alto, California); Calbiochem (San Diego, California) and Miles Laboratories (Elkhart, Indiana). In addition, TRH, LHRH and somatostatin were generously supplied by Abbot Laboratories (Chicago, Illinois) and Ayerst Laboratories (Montreal, Canada).

RESULTS

The polypeptide hormones differ in number and sequence of amino acid and absorbance. The selection of UV absorption wavelength is a function of the absorption spectra of each individual peptide. While the enkephalins, angiotensin II, DSIP, LHRH and somatostatin can be readily monitored at 254 or 280 nm, TRH does not show absorbance at these wavelengths (8). It is thus necessary to monitor TRH at 215 nm where absorption interferences limit the choice of eluting buffering materials. With the exception of TRH, we chose therefore to study all of these synthetic peptides

at 254 nm where we had a wide choice of eluting systems and to study TRH at 215 nm.

The amino acid sequences of all of the synthetic peptides studied is given in Table I. The chromatogram of leu and met-enkephalin (Figure 1) was obtained from a freshly prepared solu-

TABLE 1

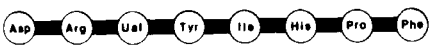
Amino Acid Sequences and Molecular Weights of the Synthetic Peptides Studied.

THYROTROPIN RELEASING HORMONE mw 361

METHIONINE-ENKEPHALIN mw574

LEUCINE-ENKEPHALIN mw556

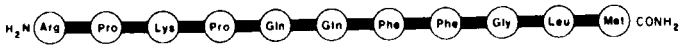
ANGIOTENSIN II mw1046



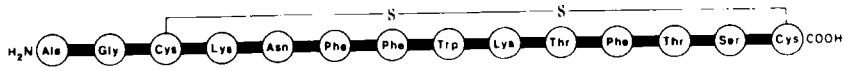
DELTA SLEEP INDUCING HORMONE (DSIP) mw848.98

LUTEINIZING HORMONE RELEASING HORMONE mw1182.5

SUBSTANCE P mw1347



SOMATOSTATIN mw1639



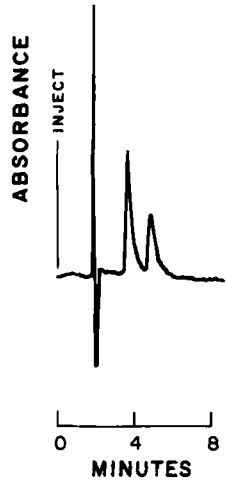


FIGURE 1

Separation of leu and met enkephalin. UV detector 254 nm, 0.04 AUFS. Mobile phase 40% methanol in 8% acetic acid. Flow rate 1.5 ml/min. Compound 1 - met-enkephalin; 2-leu-enkephalin.

tion separated under isocratic conditions by elution from a Fatty Acid Analysis^{R} Column. The elution time for met-enkephalin was 3.45 minutes and for leu-enkephalin 4.52 minutes. The type of tailing observed with both peptides probably represents shoulder impurities in the synthetic material. Although these two neuropeptides differ by only one amino acid in their chain sequence, the baseline resolution between them permits their ready detection and quantification. A solution of leu-enkephalin stored for 120 days in a refrigerator at 8°C under sterile conditions was chromatographed under identical conditions and showed at least two major peaks and some minor components (Figure 2). All of the peptides stored under normal refrigeration showed aqueous solution instability except LHRH which remained stable for a period of 6 months.

A freshly prepared solution of angiotensin II (Beckman) indicates a degree of impurity with at least seven identifiable peaks (Figure 3). The retention time of the major peak was 4.22 minutes.

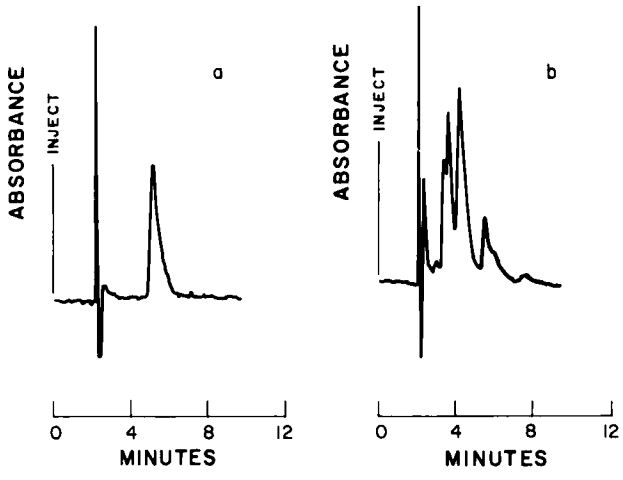


FIGURE 2

Chromatogram of an aqueous solution of leu-enkephalin. (a) freshly prepared solution; (b) solution stored at 8°C for 120 days. Conditions identical to Figure 1.

LHRH chromatographed under the identical conditions as angiotensin II shows a shoulder on the major absorbance peak (Figure 4). If methanol concentration is decreased from 50 to 40 percent the minor component can be further resolved from the major peak. Increasing column retention by decreasing methanol concentration produces a greater although not complete separation of the impurities from the major material. As shown in Figure 5, a linear response was obtained by comparing either peak height or peak area to the concentration of LHRH. Hence one can calculate the concentration of LHRH in unknown solutions following either of these two relationships. The sensitivities of this method permit measurement of nanomolar quantities of LHRH.

Because substance P has a lower molar absorptivity than the other peptides, higher concentrations are required to generate the same degree of absorbance. The chromatogram of freshly prepared substance P was compared to that of a sample stored for thirty days.

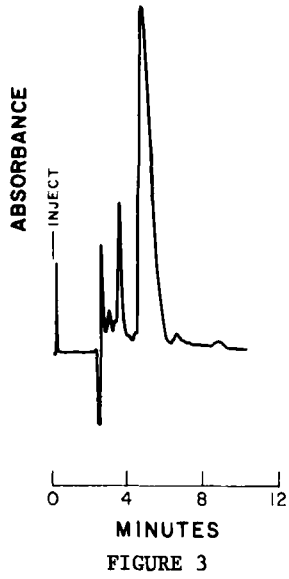


FIGURE 3

Chromatogram of an aqueous solution of angiotensin II. UV detector 254 nm; .01 AUFS mobile phase 50% methanol in 8% acetic acid. Flow rate 1.5 ml/min.

Both the stored and freshly prepared sample were chromatographed sequentially on the same day under identical conditions and revealed considerable chromatographic variation (Figure 6). The peaks appearing in the stored sample, if they follow normal reversed phase chromatography behavior, were more polar in nature since they elute before the characteristic substance P peak.

An aliquot of DSIP was graciously given to us for analysis by Professors M. Monnier and G.A. Schoenenberger and compared to the commercially obtained synthetic preparation of Calibiochem Company (Figure 7). While the two samples were chromatographed under the same conditions, on the same column, they were not analyzed on the same day. Nonetheless, the differences between the two samples can be readily observed (Figure 7). The retention time for the major peak was 4.45. The synthetic nonopeptide was chromatographed at

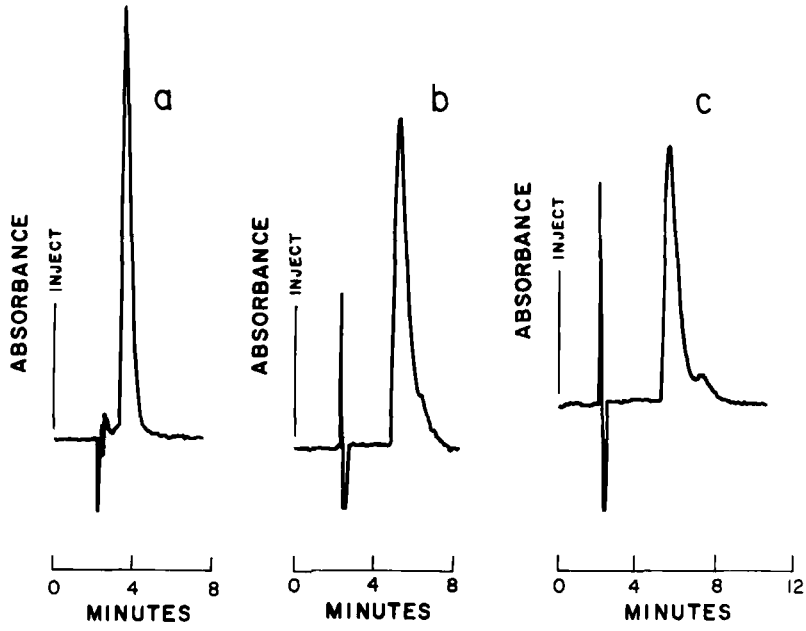


FIGURE 4

A chromatogram of an aqueous solution of LHRH showing the presence of a contaminant. The effect of the methanol concentration on retention time and resolution of the contaminant is shown. A-60, B-50, C-40 percent methanol, in mobile phase 8% acetic acid. UV detector 254 nm. AUFS .04. Flow rate 1.5 ml/min.

ten times the sensitivity as the tracing shown previously. Figure 7 clearly demonstrates the minor absorbance contaminants.

A freshly prepared solution of somatostatin and a sample stored in the refrigerator were chromatographed. The two major peaks observed in most synthetic peptide samples were observed in the fresh samples. Freshly prepared somatostatin demonstrated a major component and a minor component (Figure 8). The ratio of these two peaks can be readily altered by 3 days of storage.

We believe that this represents a reduction of the disulfide bond. Support for this hypothesis was the observation that cyclic

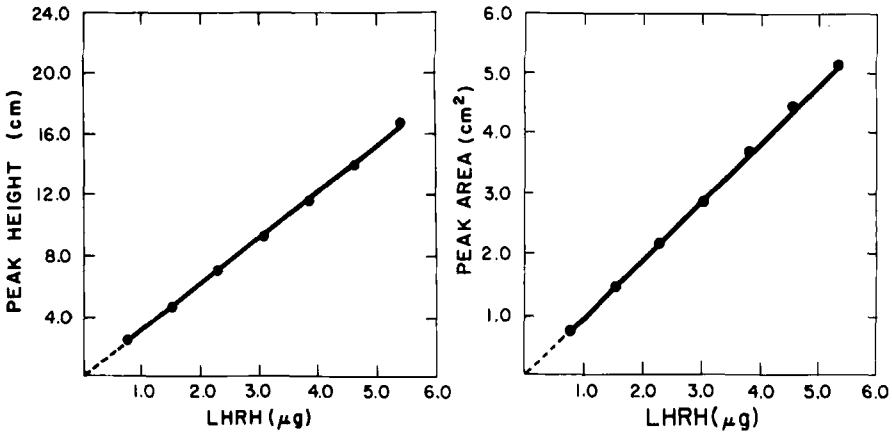


FIGURE 5

Calibration curve of LHRH; (a) peak height vs. LHRH concentration; (b) peak area vs. LHRH concentration.

somatostatin obtained from Peninsula Laboratories has a single peak corresponding to the first major peak observed in the previous figure (Figure 9). We believe, therefore, that the second peak seen when somatostatin was stored was most likely due to a reduction of the disulfide bond in this peptide.

Lacking a tyrosine and tryptophan chromaphore, TRH does not absorb at 254 nm. Acetic acid at 0.1% does not interfere with the detection of TRH at 215 nm. Using a Fatty Acid Analysis Column and an eluting system of 50% MeOH/50% HAC (0.1%), a K' of 1.23 was obtained for TRH (Figure 10). These solvent conditions are not useful for the analysis of the other peptides since they are all retained for more than 30 minutes. Like the other peptides, TRH stored twenty-seven days under refrigerated conditions lacked stability and multiple peaks were observed on the chromatogram (Figure 10).

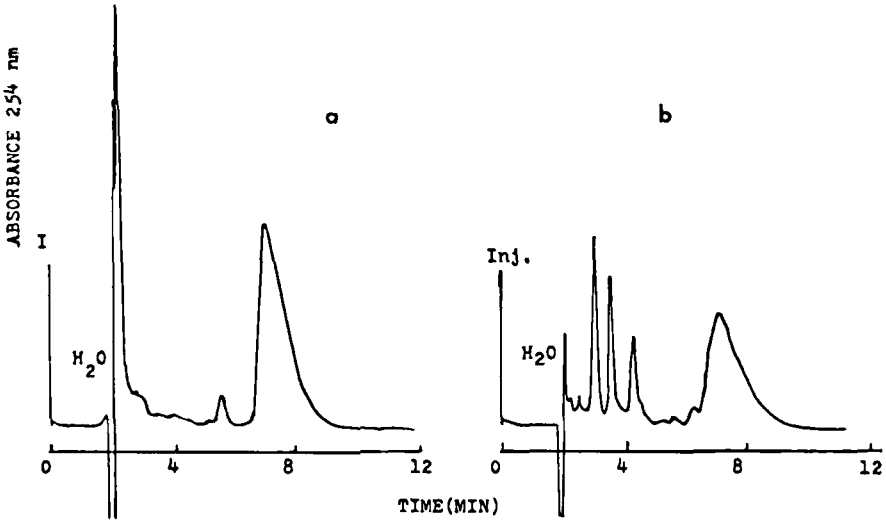


FIGURE 6

Chromatograms of fresh and stored aqueous solutions of substance P; (a) fresh preparation; (b) preparation stored at 8°C for thirty days. Mobile phase 50% methanol in 8% acetic acid. UV detector 254 nm; .005 AUFS. Flow rate 1.5 ml/min.

The retention times, peak areas and shape of the curves of all eight peptides studied were reproducible and the column stability was maintained for at least six months. While retention characteristics may vary from column to column, the conditions for analysis are readily adjustable to each new column.

DISCUSSION

Our HPLC technique had provided us with several significant advantages. The technique enables us to separate peptide molecules differing by only one amino acid . . . angiotensin II, DSIP and substance P . . . as well as peptides of similar chain lengths but varying by only one amino acid in the peptide sequence . . . leu and met enkephalin. Significantly, our method of reverse phase separation utilized nonderivatized peptides, thus avoiding one

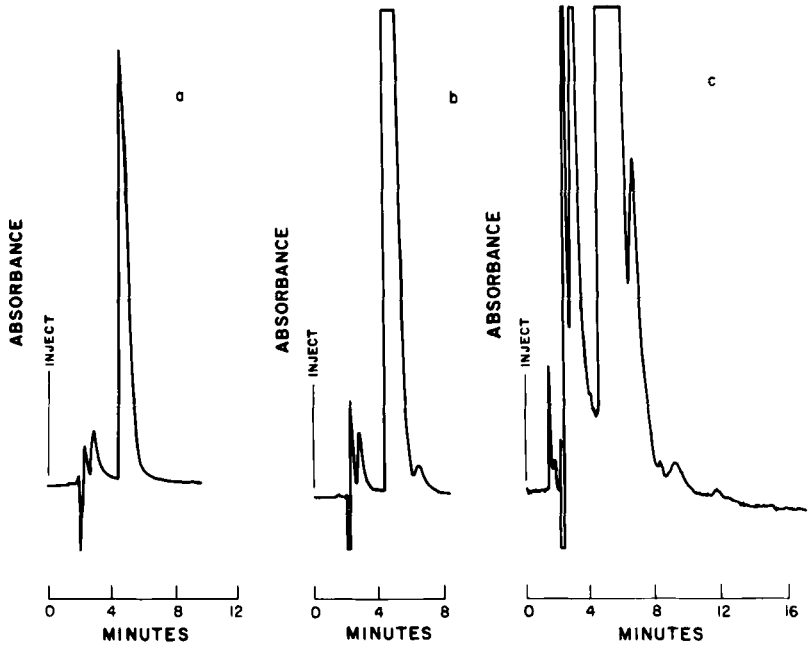


FIGURE 7

Chromatograms of aqueous solutions of DSIP; (a) Calbiochem (b) Monnier and Schoenenberger; UV detector 215 nm, 0.2 AUFS. Mobile phase 70% methanol in 0.1 % acetic acid. Flow rate 1.5 ml/min; (c) Monnier and Schoenenberger; conditions identical to the above except 0.02 AUFS.

significant area of artifact formation. By avoiding the use of derivatized peptides we have side stepped introduction of a serious source of errors into our method.

Quantification can be achieved from the peak height or peak area measurement. As a result of our techniques, we have been able to resolve, detect and quantify LHRH in human male serum (8). Analyses are in progress to study this and other peptides in blood, brain and neural tissues of animals and man.

Another benefit of our technique is that it is sufficiently refined to detect contaminants undetected by either thin layer

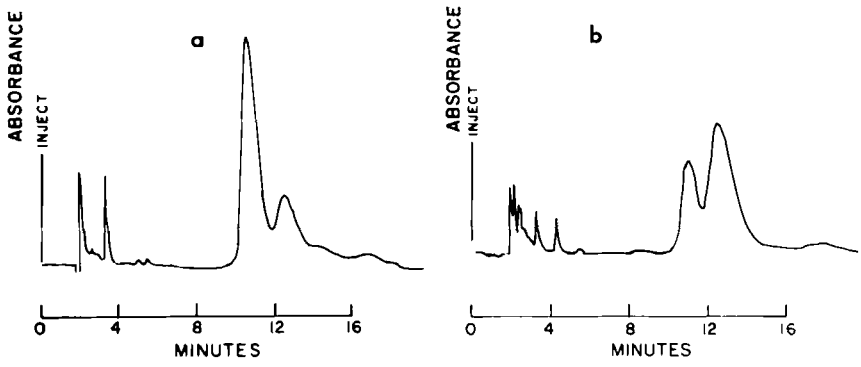


FIGURE 8

Chromatograms of (a) freshly prepared and (b) stored aqueous solutions of somatostatin. UV detector 254 nm; freshly prepared sample 0.05 AUFS; stored sample 0.01 AUFS. Mobile phase 50% methanol in 8% acetic acid. Flow rate 1.5 ml/min.

chromatography or ion exchange chromatography. Since commercially available peptides are rarely 100% pure, contamination remains a major problem. Indeed, it has recently been shown that trace contamination of a nucleotide produced significant changes in biological activity when compared to a pure substrate (9). Immunologic analytic procedures which rely on impure peptides for antibody formation lead to spurious results.

It is indisputable that a method to obtain analytic pure materials is desirable. HPLC allows for a significant purification of synthetic peptides. It should be pointed out, however, that detection of impurities depends on monitoring at more than one wavelength. Scanning detectors may serve a significant role in the measurement of purity as well as the identification of unknown peptide material.

Some questions may arise as to the cause of peptide degradation. Although it is commonly believed that the peptide linkage is stable in aqueous solution, we have clearly demonstrated that most peptides are unstable when stored at 8°C. We do not believe that these changes are the result of polymerization, since under reversed

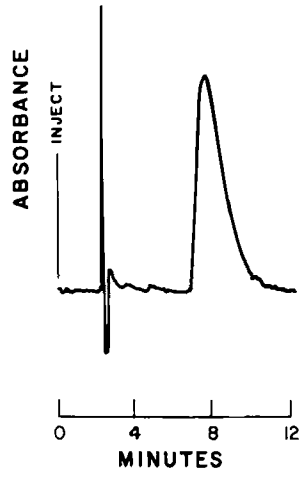


FIGURE 9

Chromatogram of somatostatin freshly prepared showing a single peak. Conditions the same as for Figure 8. 0.02 AUFS.

phase separation conditions polymers have prolonged retention times due to their lipophilic nature. Since our peptides were not stored under anaerobic conditions, oxidation might have occurred. But the multiplicity of the fragments suggests that hydrolysis occurred at many sites and in a random manner. Since biological tissue exists in an aerobic condition, specific proteolytic enzymes may not be required to obtain degradation of these peptides. We are currently studying these synthetic neuroendocrine peptides in extracts of blood and brain tissues to determine their degradative products.

A positive benefit of our system is its rapidity. By varying the reversed phase solvent systems, under isocratic conditions we can separate peptides in minutes. This is remarkable considering the variety of polarities and similarities of structure of the peptides we studied. Met and leu enkephalin, angiotensin II and DSIP, for example, have free terminal amino acid groups, while TRH and LHRH do not. Also, since we do not employ gradient conditions,

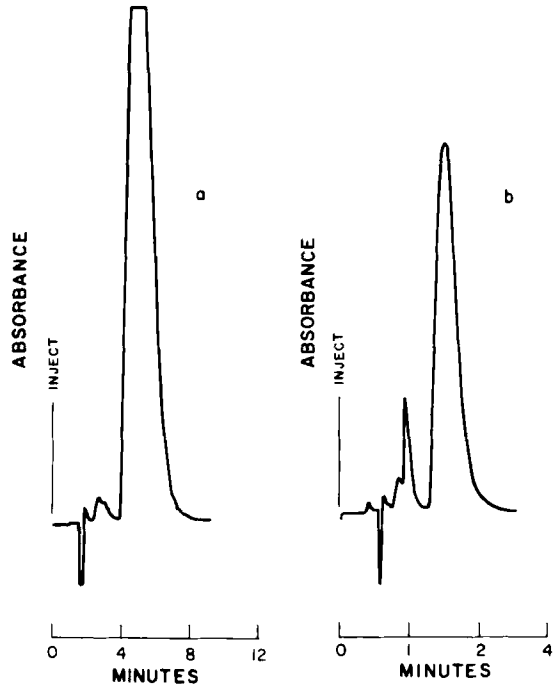


FIGURE 10

Chromatograms of TRH (a) freshly prepared and (b) stored aqueous solutions. UV 215 nm, 0.2 AUFS mobile phase 60% methanol in 0.1% acetic acid. Flow rate 1.5 ml/min.

a column regeneration step after each analysis is unnecessary. While we have demonstrated the separation only in the nonpolar Fatty Acid Analysis Column, we have elsewhere reported other column conditions and other solvent systems with some of these peptides (8). But in any case it is clear that the HPLC separation of the peptides and their degradation products for analysis and quantification open new paths for the study of the purity, stability and biologic roles of the peptides.

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