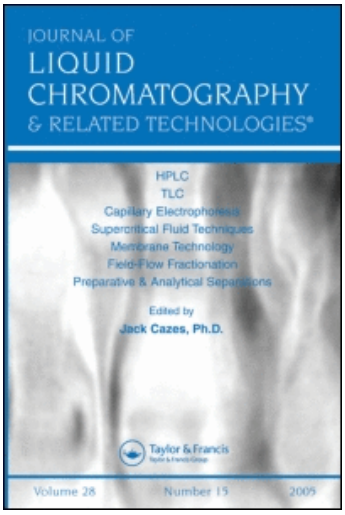

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High Performance Liquid Chromatography of Enkephalin and Endorphin Peptide Analogs

Bruce L. Currie[a]; Jaw Kang Chang[b]; Robert Cooley[c]
[a] Department of Medicinal, Chemistry University of Illinois at the Medical Center Chicago, Illinois [b] Peninsula Laboratories, San Carlos, California [c] Waters Associates, Inc., Milford, Massachusetts

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ENKEPHALIN AND ENDORPHIN PEPTIDE ANALOGS

Bruce L. Currie*
Department of Medicinal Chemistry
University of Illinois at the Medical Center
Chicago, Illinois 60680

and

Jaw - Kang Chang
Peninsula Laboratories, P. O. Box 1111
San Carlos, California 94070

and

Robert Cooley
Waters Associates, Inc., Maple Street
Milford, Massachusetts 01757

ABSTRACT

Reverse phase systems are presented which utilize a μ alkylphenyl column and ammonium acetate buffered aqueous acetonitrile mobile phases to separate mixtures of enkephalin and endorphin peptide analogs. High pressure liquid chromatographic separations of mixtures of enkephalin diastereomers as well as mixtures of other closely similar analogs have been developed.

Endorphin analogs were observed to be quite hydrophobic and required mobile phases containing 40% or more acetonitrile for reasonable elution times. The enkephalin analogs by comparison required 20% or more acetonitrile. Detection at both 254 and 280 nm was useful in recognizing the important peaks in the elution profile.

INTRODUCTION

Methionine-enkephalin, leucine-enkephalin and the endorphin family of peptides have recently been isolated from the brain and other tissues of mammals (1-3). These peptides have opiate-like biological activity as well as other effects on the central nervous system (4). Elucidation of the sequence of these peptides brought the realization that the entire sequence of methionine-enkephalin is homologous to the amino terminal portion of the endorphins. The endorphin structures have been found within the carboxyl terminal portion of the pituitary hormone, β-lipotropin, and thus, are presumed to arise physiologically from β-lipotropin.

The role of these peptides as components of a natural pain control system has generated much recent interest in the study of the pharmacological properties of this class of peptides. Many synthetic analogs have been prepared in order to gain a better understanding of the functional role of these peptides and to develop more potent, and possibly oral activity as analgesic agents, and that may have less dependence liability than the opiate alkaloids.

This paper reports the development of HPLC systems that can be used to evaluate the purity of these types of synthetic peptides and that may be useful also as

purification methods. Also reported are isocratic systems for the resolution of diastereomeric pairs of enkephalins as well as other closely similar analogs.

MATERIALS

All of the peptides in Tables 1 and 2 are commercially available samples from Peninsula Laboratories. All reported separations were conducted on a μBONDAPAK/Phenyl column, 3.9 mm ID x 30 cm, 10 μ particle size. Acetonitrile was glass distilled, "UV grade" from Burdick and Jackson Laboratories (Muskegan,Michigan). Water was glass distilled and filtered through a 0.22 μ membrane. Peptide samples were dissolved in glass distilled water at a concentration of 1 mg/10 ml and stored frozen when not in use. Ammonium acetate and acetic acid were analytical reagent grade.

METHODS

A Waters Associates, Inc. (Milford, Massachusetts) M-6000 solvent delivery system was used to deliver a flow rate of 1.0 ml/min of the mobile phases reported in Tables 1 and 2. All solvent systems were used isocratically at ambient temperature (22-24°C). Peptide samples in distilled water were injected through a white silicon rubber (WSR) septum with a Precision Sampling Corporation Pressure-Pak liquid syringe series

TABLE 1

CAPACITY FACTORS FOR ENKEPHALIN ANALOGS ON μBONDAPAK/PHENYL

PEPTIDES	Percent Acetonitrile[+]				
	25	30	45	50[++]	55
Tyr-D-Ala-Gly-Phe-Met-NH_2 (1)	2.54	1.94	1.77	2.07	1.45
Tyr-D-Ala-Gly-Phe-Met (2)	1.37	1.26			1.25
Tyr-D-Ala-Gly-Phe-Leu-NH_2 (3)	3.15	2.38	2.00	1.64	1.33
Tyr-Gly-Gly-Phe-Leu-NH_2 (4)	2.75	1.95	1.77	2.00	1.70
Tyr-Gly-Gly-Phe-Leu (5)	1.30	1.17			1.15
Tyr-D-Met-Gly-Phe-Pro-NH_2 (6)	3.40	2.32	2.00	2.21	
Tyr-2-MeAla-Gly-Phe-Met-NH_2 (7)	3.35	2.25	2.00	2.21	
Tyr-Gly-Gly-Phe-Met-NH_2 (8)	2.20	1.69			
Tyr-Ala-Gly-Phe-Met-NH_2 (9)	2.35	2.10	1.85	2.15	
Tyr-Gly-Gly-Phe-Met (10)	1.21	1.16			
3,5-Br_2-Tyr-Gly-Gly-Phe-Leu (11)	1.97	1.80			
Ala-Ala-Ala-Tyr-Gly-Gly-Phe-Leu (12)	1.47	1.40			
Ala-Ala-Ala-Tyr-Gly-Gly-Phe-Met (13)	1.16	1.11			
3,5-Br_2-Tyr-Gly-Gly-Phe-Met (14)	1.94	1.56			

[+] The mobile phase consists of aqueous acetonitrile containing 0.01 M NH_4OAc adjusted to pH 4.5 with HOAc.

[++] The mobile phase consists of aqueous acetonitrile containing 0.005 M NH_4OAc adjusted to pH 4.5 with HOAc.

TABLE 2

CAPACITY FACTORS FOR ENDORPHIN ANALOGS ON μBONDAPAK/PHENYL

PEPTIDES	Percent Acetonitrile[+] 40	45	50[++]
D-Ala^2-Beta-Endorphin (Human) (15)	3.09	2.69	1.85
Gamma-Endorphin (β-Lipotropin 61-77) (16)	2.18	1.15	1.08
Alpha-Endorphin (β-Lipotropin 61-76) (17)	1.94	1.15	1.00
2-MeAla^2-Beta-Endorphin (Human) (18)	3.27	3.08	1.92
Arg-Beta-Endorphin (Human) (19)	2.67	2.46	--
Beta-Endorphin (Human) (β-Lipotropin 61-91) (20)	2.26	1.82	--
Beta-Endorphin (Camel) (21)	2.76	2.55	--

[+] The mobile phase consists of aqueous acetonitrile containing 0.01 M NH_4OAc adjusted to pH 4.5 with HOAc.

[++] The mobile phase consists of aqueous acetonitrile containing 0.005 M NH_4OAc adjusted to pH 4.5 with HOAc.

B-110 (Baton Rouge, Louisiana). A Waters-Beckman LC-25 at 280 nm, 0.1 aufs, and a Waters Associates Model 440 at 254 nm, 0.01 aufs, connected in series, were used as detectors. Chart speed was 6"/hr.

RESULTS

Twelve synthetic analogs of either methionine-enkephalin or leucine-enkephalin as well as synthetic samples of the two natural enkephalins,and seven peptides of the endorphin family were the samples used in this study. The enkephalins ranged in size from five to eight amino acid residues and included a pair of diastereomers that differed only in the configuration of the amino acid at the second position. Included were seven samples that were carboxy terminal amides and seven that were carboxy terminal acids. All fourteen samples had free terminal amino groups. The endorphin analogs ranged in size from sixteen to thirty-two residues.

These two groups of closely similar peptides were used to evaluate the separation potential of μBONDAPAK/Phenyl for the resolution of oligopeptides. All of these samples were monitored at 254 and 280 nm by using two detectors in series. The use of ammonium acetate buffered aqueous acetonitrile mobile phases provided no significant interference at these wave-

lengths under the isocratic conditions employed in these studies.

Variation of the acetonitrile component of the mobile phase from 55% to 25% revealed the general behavior of the column as "reverse phase", since a decrease in the organic component of the solvent resulted in an increase in the retention of the sample (Table 1). This was further illustrated by the observation that the peptides with free carboxylic acid terminal groups were less retained than the peptide of the same sequence except having a carboxy terminal amide.

It was apparent, also, that the retention of the peptides was sensitive to changes in the molarity of the buffer component of the mobile phase as can be seen from the data in Tables 1 and 2. A decrease in the NH_4OAc concentration generally resulting in an increased retention of the peptides.

Most of the peptide samples consisted of one peak or one major peak (>90%) at 280 nm, while at 254 nm one or more additional peaks may be apparent usually close to the solvent front. These may be due to a small amount of solvent residue and probably do not represent peptide impurities (Figures 1,2 and 3.).

A similar sample size for both the endorphin and enkephalin analogs was used, i.e. 2.5 µg. Smaller amplitude peaks were observed for the endorphin

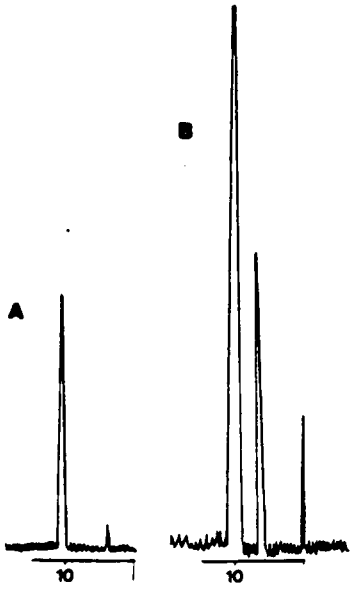

FIGURE 1: Sample, Tyr-D-Ala-Gly-Phe-Met-NH_2 (1); mobile phase, 25% aqueous CH_3CN containing 0.01 M NH_4OAc adjusted to pH 4.5; flow rate, 1 ml/min; detector, trace A, 280 nm, 0.1 aufs, trace B, 254 nm, 0.01 aufs.

samples compared to the enkephalin samples. This observation is consistent with the relatively less aromatic amino acid content for the endorphin samples compared to the enkephalin samples (Figure 6).

The resolution of the diastereomeric analogs with either L-Ala or D-Ala at the two position could be achieved using the 25% acetonitrile system. Even though the amino acid sequence of these peptides are identical with the exception of the optical configuration of the alanine residue, the resulting conformational change produces a difference in polarity that

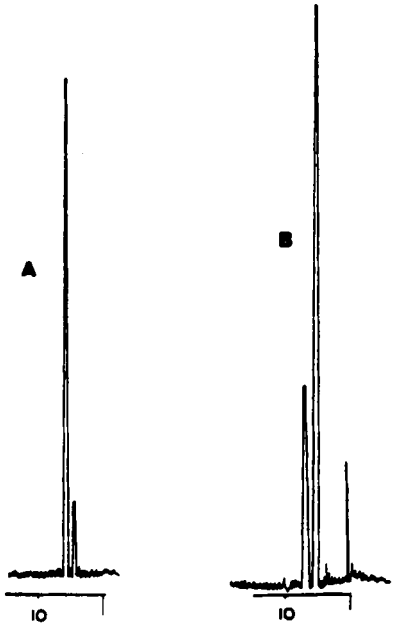

FIGURE 2: Sample, Tyr-D-Ala-Gly-Pne-Met (2); mobile phase, 25% aqueous CH_3CN containing 0.01 M NH_4OAc adjusted to pH 4.5; flow rate, 1 ml/min; detector, trace A, 280 nm, 0.1 aufs, trace B, 254 nm, 0.01 aufs.

permits the separation. The peptide containing the L-isomer eluting first followed by the D isomer. Mixtures containing the above diastereomers and also the α-methylalanine analog, 7, or the α-methylalanine analog, 7, and the natural glycine, 8, containing analog could also be separated using the 20% acetonitrile system (Figures 4 and 5.).

DISCUSSION

The results of this study show the utility of high performance liquid chromatography as a tool for

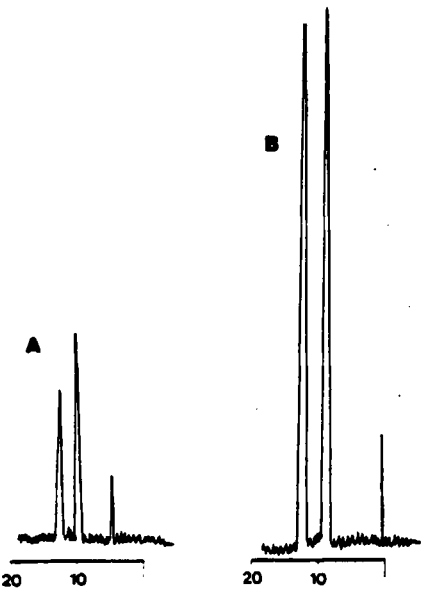

FIGURE 3: Sample, mixture of Tyr-Gly-Gly-Phe-Leu (5) and Tyr-Gly-Gly-Phe-Met (10), left to right; mobile phase, 25% aqueous CH_3CN containing 0.01 M NH_4OAc adjusted to pH 4.5; flow rate, 1 ml/min; detector, trace A, 280 nm, 0.1 aufs, trace B, 254 nm, 0.01 aufs.

the evaluation of purity of synthetic peptides and the versatility of the μBONDAPAK/Phenyl column for peptide samples ranging in size from five to thirty-two residues in length. The greater hydrophobicity of the endorphins relative to the enkephalins was apparent since at least 40% acetonitrile mixtures were required for reasonable elution times for the endorphins. The enkephalins could be eluted with similar retention times with 20% acetonitrile mixtures.

The ability to separate diastereomeric pairs of peptides is especially useful for the determination

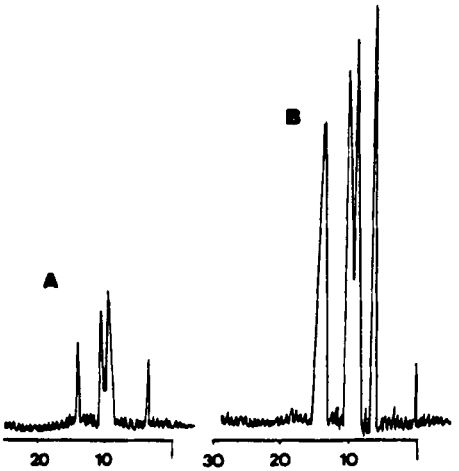

FIGURE 4: Sample, mixture of Tyr-2-Me-Ala-Gly-Phe-Met-NH_2 (7), Tyr-D-Ala-Gly-Phe-Met-NH_2 (1) and Tyr-Ala-Gly-Phe-Met-NH_2 (9), left to right; mobile phase, 25% aqueous CH_3CN containing 0.01 M NH_4OAc adjusted to pH 4.5; flow rate, 1 ml/min; detector, trace A, 280 nm, 0.1 aufs, trace B, 254 nm, 0.01 aufs.

of the extent of racemization that may occur during peptide synthesis. The resolution of diastereomers as well as the other closely similar peptides demonstrates the resolving power of this HPLC system. Previous reports have appeared in the literature concerning peptide diastereomer separations of several model dipeptides. These previous studies have utilized either microsilica (5) or alkyl silica, C_8 or C_{18} (6, 7) columns and were able to achieve baseline separation of the diastereomers under investigation. Separation of diastereomeric oxytocin peptides has recently been reported using a μC_{18} column (8). Also using C_8 and C_{18}, analytical and preparative separa-

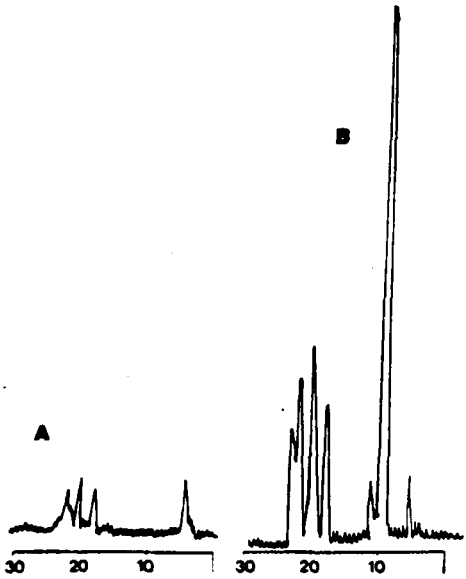

FIGURE 5: Sample, mixture of Tyr-2-MeAla-Gly-Phe-Met-NH_2 (7), Tyr-D-Ala-Gly-Phe-Met-NH_2 (1), Tyr-Ala-Gly-Phe-Met-NH_2 (9), and Tyr-Gly-Gly-Phe-Met-NH_2 (8) left to right; mobile phase, 20% aqueous CH_3CN containing 0.01 M NH_4OAc adjusted to pH 4.5; flow rate 1 ml/min; detector, trace A, 280 nm, 0.1 aufs, trace B, 254 nm, 0.01 aufs.

tions of enkephalin diastereomers were reported (9) while this manuscript was in preparation.

Recent reports have also appeared regarding HPLC of the naturally occurring enkephalins as well as other peptides and peptide hormones. The μC_{18} (10,11) and fatty acid analysis (12) columns were used in these studies. The use of trialkylammonium phosphate buffers with several column types for chromatography of peptides has also been reported (13).

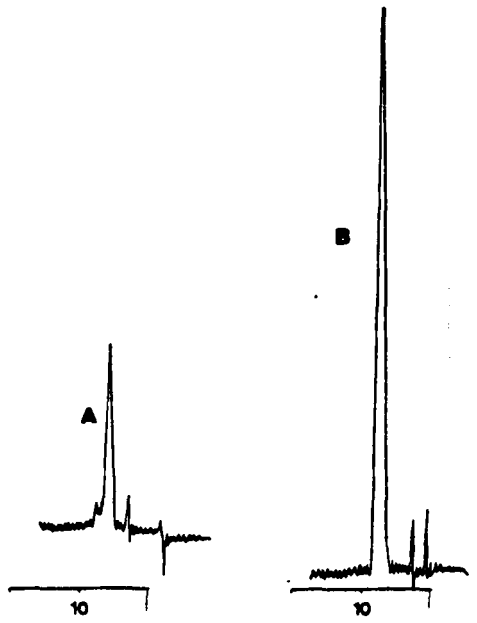

FIGURE 6: Sample, Beta-endorphin (camel) (21); mobile phase, 45% aqueous CH_3CN containing 0.01 M NH_4OAc adjusted to pH 4.5; flow rate, 1 ml/min; detector, trace A, 280 nm, 0.1 aufs, trace B, 254 nm, 0.01 aufs.

Our results with the recently introduced phenyl-silica type of column indicate that this packing material can be added to the arsenal available to the peptide chemist to be used in addressing the difficult separation problems that often occur. The addition of low concentrations of buffer to the mobile phase increases the sharpness of peaks and thus the resolving power of the solvent system and seems to be particularly important for these peptide samples. The use of both 254 nm and 280 nm wavelengths was

particularly useful in distinguishing peaks observed that were probably not peptidic or at least lacked the amino terminal tyrosine residue.

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